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# Characterization and Assessment of Virulence Factors and Antigenic Determinants of *Pseudomonas (Burkholderia) Pseudomallei (Final Report)*

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# FINAL REPORT

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## CHARACTERIZATION AND ASSESSMENT OF VIRULENCE FACTORS AND ANTIGENIC DETERMINANTS OF *PSEUDOMONAS (BURKHOLDERIA) PSEUDOMALLEI*

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# Isolation and Characterization of *Burkholderia pseudomallei* Flagellin Proteins

# A. INTRODUCTION

*Burkholderia pseudomallei*, the etiologic agent of melioidosis, is responsible for a broad spectrum of infections observed in both humans and animals (13, 52). Clinical manifestations of melioidosis may range from the chronic, sub-clinical forms to the acute septicemic and acute pulmonary presentations of the disease. In many instances the acute manifestations of melioidosis are associated with an appalling mortality rate, even when vigorous chemotheraputic intervention is implemented (38, 73). To this end, a prophylaxis measure in the form of a vaccination against *B. pseudomallei* infections would be desirable for those who reside in endemic regions or other populations that may be at particular risk of acquiring the disease. Unfortunately, however, there are currently no suitable vaccine preparations available for this undertaking.

The primary reason for the lack of a vaccination against melioidosis is that to date very little is understood regarding the pathogenic mechanisms associated with this organism. Although a number of putative virulence determinants have been identified, including secreted and cell-associated antigens, they are poorly characterized at both the immunological and biochemical levels (11, 26, 33, 34, 70, 76, 87, 88). Preliminary studies in our laboratory initiated to define the role of motility in the pathogenesis of *B. pseudomallei* infections, however, have suggested that components of the

flagellar apparatus, in particular flagellin proteins, may prove to be excellent candidates as protective antigens against melioidosis.

Bacterial flagella are organelles, expressed by both archaeobacteria and eubacteria, that enable a microorganism to respond to its immediate environment by facilitating the migration towards favorable or away from undesirable stimuli (82). Since 80% of all bacterial species studied demonstrate the presence of a flagellar apparatus, these complex structures must be an important feature for the survival of many prokaryotes in their respective environmental niches (1). It has been well documented that the flagella expressed by bacterial pathogens such as *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Vibrio anguillarum*, *Clostridium chauvoei* and *Proteus mirabilis* contribute significantly to the establishment of infections caused by these organisms (5, 56, 58, 77, 89). While the pathogenesis of many bacterial diseases can be directly attributed to the motility enhanced dissemination of bacteria from the initial site of colonization, it has also been demonstrated that proteins in the flagellar apparatus may also function as bacterial adhesins (77, 89).

Bacteria display from one to many hundred flagella in a variety of arrays depending on the particular species and the physiological composition of their surroundings (82). Morphologically, flagella consist of three major components designated the basal body, the hook structure and the filament. The filament is composed almost exclusively of polymerized flagellin protein subunits which form an elongated macrohelix. Coupling of the filament to the basal body,

through the hook, enables the flagellar filament to be rotated via a complex series of events driven either by electrochemical or proton gradients (1, 4, 32). The net result is the propulsion of the bacterium through the environmental milieu. Flagellar filaments such as those expressed by *P. aeruginosa* and *Salmonella typhimurium* consist of a single repeating flagellin subunit and are designated simple structures. In contrast, the filaments of organisms such as *C. jejuni*, *Helicobacter pylori*, *Caulobacter crescentus* and many of the Spirochetes contain multiple flagellin subunits and are referred to as complex flagella. Many of the complex macrohelicies are found to be enclosed by a membranous sheath, the functions of which are still poorly defined (82, 83). Bacterial flagella also demonstrate variation with respect to pitch, length and handedness depending upon environmental pH, osmolarity, temperature and the primary sequence of the flagellin subunits (82).

Flagellin proteins are characterized by a distinctive domainal structure consisting of highly conserved $NH_2$ - and $CO_2H$ -terminal regions separated by a hypervariable central domain. Conservation of the terminal residues is required for the proper folding, export and polymerization of the protein subunits into a mature, functional filament (45, 55). The variable central domain, in contrast, dictates the size of the flagellin molecule and contains many of the antigenic determinants associated with individual flagellin proteins. In fact, the variability of the central domain is so pronounced, even between strains of the same species, that these regions have been successfully exploited as sensitive biomarkers for epidemiological surveys and strain typing (83).

Flagellins are commonly the immunodominant antigen in bacterial infections, a feature that has made them desirable candidates for the construction of vaccines (60, 65).

In this study we describe the isolation and purification of the flagellin antigens from a number of *B. pseudomallei* strains, as well as their immunological and biochemical characterization. The purpose of these experiments was to determine the suitability of flagellin proteins for use as vaccine candidates against melioidosis.

## B. MATERIALS AND METHODS

### 1. Bacterial strains, media and growth conditions

The bacterial strains used in this study are described in Table 1. All stock cultures were maintained in either 10% skim milk or 20% glycerol suspensions and stored at -70°C. *B. pseudomallei* and *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) broth or on LB agar plates (Difco Laboratories Inc.). For animal studies, *B. pseudomallei* cultures were grown at 37°C in TSBDC media (8). When appropriate, antibiotics were added at the following concentrations; 100 mg/ml ampicillin (Ap; Sigma Chemical Co.) for *E. coli.*

### 2. Flagellin isolation and purification

Ten 2.0 L flasks, each containing 500 ml of LB broth supplemented with 0.2% glucose, were inoculated with 1.0 ml of an overnight *B. pseudomallei* culture. These were incubated with stirring at 100 rpm and 37°C for approximately 18 hours. Cells were pelleted from the culture medium via centrifugation at 7,000 x g and 4°C for 20 minutes prior to resuspention in 300 ml of 50 mM sodium phosphate buffer (pH 7.0). The cell suspension was blended on the low setting in a Waring commercial blender for 2 minutes in order to detach the flagellar filaments from the cells. After blending, the

homogenate was subjected to centrifugation at 9,000 x g and 4°C for 20 minutes to remove the cell bodies from the supernatant. The supernatant fraction was then saturated to 5% with ammonium sulphate and allowed to stir at room temperature for 4-5 hours prior to centrifugation at 12,000 x g for 30 minutes to remove insoluble material. The resulting supernatant fraction was subsequently saturated to 20% with ammonium sulphate and allowed to stir gently overnight at ambient temperature. The supernatant at this point was centrifuged at 12,000 x g and 4°C and the pelleted material saved. After resolubilization of the pellet in a minimum of $dH_2O$, the crude flagellin preparation was reduced to pH 2.7 via the dropwise addition of 2.0 M citric acid and stirred at 4°C for 10-15 minutes. The preparation was then subjected to ultracentrifugation at 100,000 x g and 4°C for 1 hour. Following the centrifugation, the supernatant was filtered through a 0.22 μm syringe filter and then restored to pH 7.0 via the dropwise addition of 2.0 M NaOH. After dialysis against $dH_2O$ for 24 hours, the purified flagellin protein was quantitated using the bicinchoninic acid (BCA) protein assay (Pierce Chemical Co.).

### 3. Electrophoresis and immunoblotting

Purified antigens or whole cells were mixed with sodium dodecyl sulphate (SDS) sample buffer, boiled for 5 minutes and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using either the mini (Bio-Rad Laboratories) or standard slab gel systems (51). The

separated proteins were visualized with Coomassie Brilliant Blue R-250 (CBB R-250; Bio-Rad Laboratories). Electrophoretic transfer of antigens to nitrocellulose membrane was performed as described by Towbin *et al* (79) using a Bio-Rad Trans-Blot Cell (Bio-Rad Laboratories) operating at 80-90 V for 3-4 hours and 4°C. Western immunoblotting was performed as described by Catty (12). Primary antibodies were labeled with either goat anti-rabbit IgG horse radish peroxidase (HRP) or goat anti-mouse IgM HRP conjugates at a concentration of 1:1000. Immunoblots were developed with HRP Color Development Reagent ( Bio-Rad Laboratories ).

**4. $NH_2$ -terminal sequencing**

Purified samples of *B. pseudomallei* 319a flagellin protein were subjected to SDS-PAGE (4% stacking/12.5% separating), electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane and stained with a modified CBB R-250 solution. The flagellin band was excised, and the $NH_2$ -terminal amino acid sequence was determined by S. Kielland at the Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada, employing standard Edman degradation techniques.

## 5. Amino acid composition

A vacuum-dried sample containing approximately 7 μg of *B. pseudomallei* 319a flagellin protein was mixed with 5 nmol of norleucine and hydrolyzed with 6 N hydrochloric acid (HCl) plus 0.1% ß-mercaptoethanol for 1 hour *in vacuo* at 150°C. The samples were dried and redissolved in sample buffer for analysis. Cysteine content was analyzed by performic acid hydrolysis, and tryptophan residues were assessed by tryptophan HCl hydrolysis. The amino acid composition analysis was performed by D. McKay at the Protein Sequencing Facility located at the University of Calgary Heaith Sciences Centre, Calgary, Alberta Canada.

## 6. Antibody production

Antibodies to *B. pseudomallei* 319a flagellin protein were prepared by administration of Freund's complete adjuvant emulsified with purified 319a flagellin protein in 10 mM phosphate buffered saline (PBS). The rabbits (New Zealand White, 2-2.5 kg) each received an intramuscular injection of 0.50 ml of the emulsion in both their right and left hind thigh muscles. Each injection contained approximately 100 μg of protein per ml. The rabbits were subsequently boosted at 14 day intervals using Freund's incomplete adjuvant as the emulsifying agent and 100 μg of antigen per ml. Antiserum demonstrated a high titre against purified flagellin at 42 days post

immunization as demonstrated by ELISA and thus the rabbits were exsanguinated by cardiac puncture under anesthesia (antiserum titre > $1/10^6$). The serum fraction was obtained from the whole blood and stored in aliquots at -70°C until required for use.

## 7. Isolation of immunoglobulin-G from rabbit antiserum

An Affi-Gel Protein A MAPS II kit was used to isolate the immunoglobulin-G (IgG) fraction from the rabbit polyclonal antiserum raised against the 319a flagellin protein (Bio-Rad Laboratories).

## 8. Motility inhibition assays

Two rows (6 wells per row) from a 24-well microtitre plate were filled with 1.5 ml of motility agar per well (LB broth plus 0.3% agar) containing various dilutions of Type II lipopolysaccharide (LPS) specific monoclonal antibody (MAb) (11) in the order of 0, 1:10, 1:100, 1:1000, 1:5000. These were designated control lanes. The remaining two rows were filled with the same volume of motility agar, however, they contained dilutions of the anti-319a polyclonal rabbit serum in the order of 0, 1:10, 1:100, 1:1000, 1:5000 and were designated the antibody containing lanes. One control row and one antibody containing row were inoculated with *P. aeruginosa* DG1, and one control and one antibody containing row were inoculated with *B. pseudomallei* 319a. The

plate was incubated for 12 hours at 37°C. Following incubation, the wells were examined for signs of motility as demonstrated by a halo originating from the center of the inoculum stab.

## 9. Animal protection studies

Passive immunoprotection studies were performed in the diabetic infant rat animal model of infection as previously described (85). One half of the animals serving as unimmunized controls received 100 μl of a 15 mg/ml bovine serum albumin (BSA)-PBS solution by intraperitoneal injection (i.p.). The remainder of the animals were passively immunized i.p. with 100 μl aliquots of a 15 mg/ml PBS solution of MAPS II purified antiflagellin IgG. Five animals in each of the control and immunized groups were inoculated i.p. with one of four doses of *B. pseudomallei* 316c: $10^3$, $10^4$, $10^5$ and $10^6$. Animals were monitored carefully for 6 consecutive days for signs of morbidity and mortality. Fifty-percent lethal doses ($LD_{50}$s) were calculated by the method of Reed and Muench (66). In an alternative experiment, animals were passively immunized via intravenous injection (i.v.) of the tail vein and compared to unimmunized controls in a similar manner following a *B. pseudomallei* 316c challenge.

## 10. Plasmids, primers and enzymes

The plasmids and primers used in this study are described in Table 2. Plasmids were purified from overnight cultures using Wizard™ Plus Minipreps (Promega). Restriction enzymes and T4 DNA ligase (Boehringer Mannheim) were used according to standard cloning procedures (68). DNA fragments used in cloning procedures were excised from agarose gels and purified with the GeneClean II Kit (BIO 101). Ligation mixtures were electroporated into *E. coli* DH5α using a GenePulser II/Pulse Controller Plus apparatus following the supplied protocol (Bio-Rad Laboratories).

## 11. PCR amplification of cloned *fliC*

The plasmid pPBS2H was used as a template for the polymerase chain reaction (PCR)-amplification of the *B. pseudomallei* 1026b the *fliC* gene. The oligodeoxyribonucleotide primer pair used for PCR was designed based on the previously determined 1026b *fliC* gene sequence (19). The *fliC* locus was PCR-amplified in a 100 µl reaction mix containing 10 ng of plasmid DNA, 1X PCR Buffer II, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each primer and 0.5 U of Taq DNA Polymerase (Gibco Canada Inc.). The PCR mixture was transferred to a GeneAmp PCR System 9600 (Perkin Elmer Cetus) thermal cycler and subjected to the following conditions: 97°C for 4 minutes; 30 cycles

of 97°C for 45 sec, 55°C for 45 sec, 72°C for 90 sec. The size and purity of the predicted 1.2 kb PCR product was assessed by electrophoresis of 20 µl of the reaction mixture through a 1.0% agarose gel followed by visualization of ethidium bromide-stained fragments with a UV light source. The PCR product was isolated from the reaction mixture with the Magic PCR Preps DNA Purification System (Promega).

## 12. Cloning of *fliC* PCR product

In order to express recombinant flagellin protein (rFliC) in an *E. coli* background, the PCR product was subcloned into the pUC derived pTrcHis C expression vector (Invitrogen). The purified PCR product and pTrcHis C were double digested with *Kpn*I and *Eco*RI restriction endonucleases and purified using the GeneClean II Kit. The restriction products were ligated to one another in a 20 µl reaction volume with T4 DNA ligase at 16°C overnight. Electrocompetent *E. coli* DH5α were transformed with 2 µl of the ligation mixture and grown on selective media for 36 hours. Antibiotic resistant clones were subsequently screened for plasmids containing the 1.2 kb PCR product. The resulting construct was named pTHF-CK/E.

## 13. Expression and purification of rFliC

Recombinant flagellin was expressed in *E. coli* DH5α as outlined in the Xpress™ System Protein Expression TrcHis manual (Invitrogen). The rFliC fusion protein was purified under denaturing conditions from the insoluble cell fraction via nickel chelate chromatography as described in the Xpress™ System Protein Purification manual (Invitrogen).

## 14. DNA sequencing

Automated DNA sequencing was performed by the University Core DNA Services (University of Calgary) with the ABI PRISM DyeDeoxy Termination Cycle Sequencing System and AmpliTaq DNA polymerase (Perkin-Elmer). DNA sequencing reactions were analyzed using an ABI 373A DNA Sequencer. The pTrcHis Forward Sequencing Primer (Invitrogen) was used to confirm the desired sequence of the 5'-end of pTHF-CK/E vector construct.

Table 1. Bacterial strains used in flagellin purification studies.

| Strain | Description | Source/Reference |
|---|---|---|
| *E. coli* | | |
| DH5α | F⁻ϕ80d*lacZ*ΔM15Δ( *lacZYA-argF* )U169 *endA*1 *recA*1 *hsdR17 deoR thi*-1 *supE*44 *gyrA*96 *relA*1 | Bethesda Research Laboratories |
| *P. aeruginosa* | | |
| DG1 | | |
| *B. pseudomallei* | | |
| 304f | Clinical isolate | D. A. B. Dance |
| 307d | Clinical isolate | D. A. B. Dance |
| 316c | Clinical isolate | D. A. B. Dance |
| 319a | Clinical isolate | D. A. B. Dance |
| 415d | Clinical isolate | D. A. B. Dance |
| 1026b | Clinical isolate | D. A. B. Dance |

Table 2. Plasmids and primers used in flagellin purification studies.

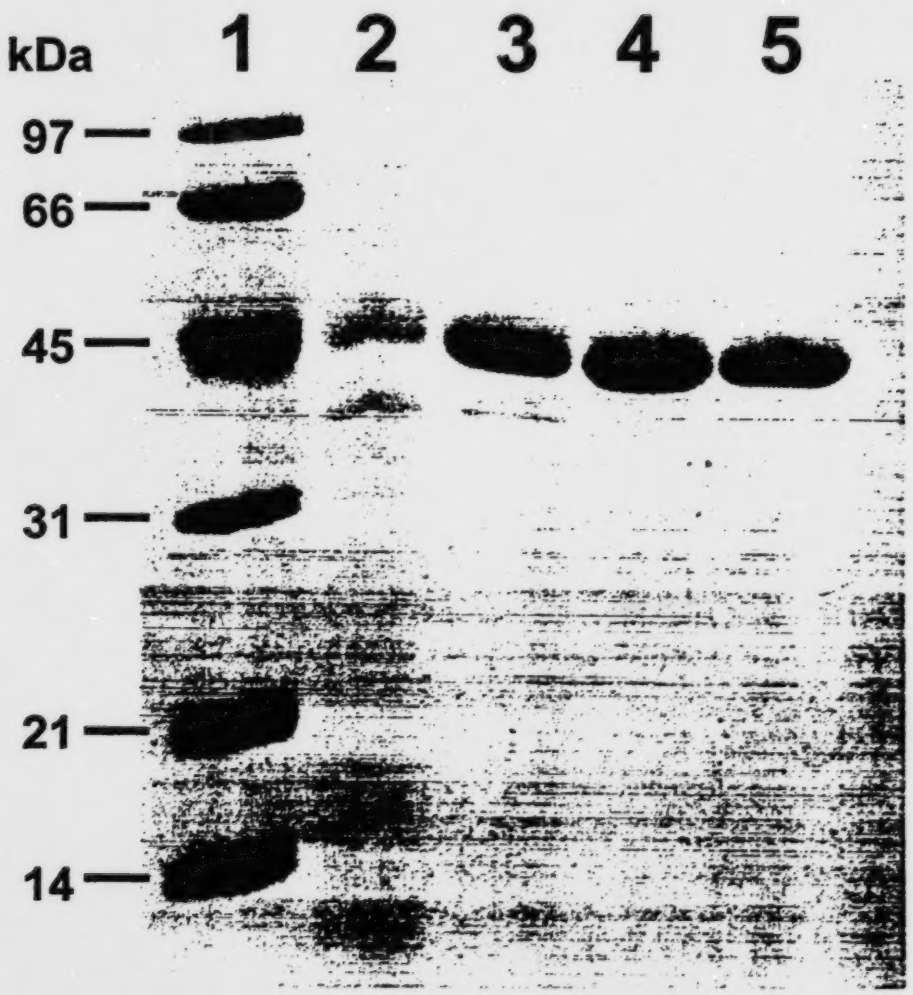
| Plasmid/Primer | Description | Reference/Source |
|---|---|---|
| pPBS2H | 8.0 kb *Hind*III fragment from PBS1 obtained by self-cloning; $Ap^R$ $Tc^R$ | (19) |
| pTrcHis C | pUC-derived expression vector; ColE1 ori; $Ap^R$ | Invitrogen |
| pTHF-CK/E | pTrcHis C containing the 1.2 kb KpnI/*Eco*RI digested PCR product | This study |
| FliC-left | 5'-GGGGTACCGCTCGGAATCAA CAGCAACATT-3' | This study |
| FliC-right | 5'-CGGAATTCTTATTGCAGGAGC TTCAGCAC-3' | This study |
| pTrcHis forward | 5'-GAGGTATATATTAATGTATCG-3' | Invitrogen |

## C. RESULTS

### 1. Flagellar filament isolation and purification

The flagellar filaments of a number of *B. pseudomallei* strains were isolated and purified to homogeneity via the application of mechanical shearing, ammonium sulphate precipitation and differential centrifugation techniques. Following mechanical shearing, the incremental addition of ammonium sulphate to crude flagellar preparations enabled relatively pure flagella to be recovered from fractions saturated to 10-20% with the precipitant (Fig. 1). Proteins precipitating below these ammonium sulphate concentrations were primarily cell debris contaminants. Following the ultracentrifugation acid-disassociation differential centrifugation-reassociation step (54), homogeneous preparations of *B. pseudomallei* flagellin proteins were acheived. Analysis of the 319a sample by SDS-PAGE demonstrated the presence of a single flagellin protein with an apparent relative mobility ( $M_r$ ) = 43,400. Overloading of the polyacrylamide gel with 50 μg of protein served to further confirm the purity of the sample (Fig. 2). Comparison of the 319a protein with flagellins isolated from strains 307d, 316c and 1026b demonstrated that the $M_r$ of subunits isolated from the four different strains of *B. pseudomallei* were indistinguishable from one another when analyzed by SDS-PAGE (Fig 3). Protein quantitation of the purified flagellin samples using the BCA assay determined that optimal yields of flagella were obtained from

Figure 1. SDS-PAGE analysis of *B. pseudomallei* 319a flagellin proteins during ammonium sulphate precipitation. Proteins were denatured in sample buffer, electrophoresed on a 4% stacking and 12.5% separating polyacrylamide mini gel apparatus and stained with CBB R-250. Lane 1, standard protein markers (rabbit muscle phosphorylase b [97.4 kDa], bovine serum albumin [66.2 kDa], hen egg white ovalbumin [45.0 kDa], bovine carbonic anhydrase [31.0 kDa], soybean trypsin inhibitor [21.5 kDa] and hen egg white lysozyme [14.4 kDa]; Bio-Rad Laboratories); lane 2, 5% ammonium sulphate fraction (5μg); lane 3, 10% ammonium sulphate fraction (5μg); lane 4, 15% ammonium sulphate fraction (5μg); lane 5, 20% ammonium sulphate fraction (5μg).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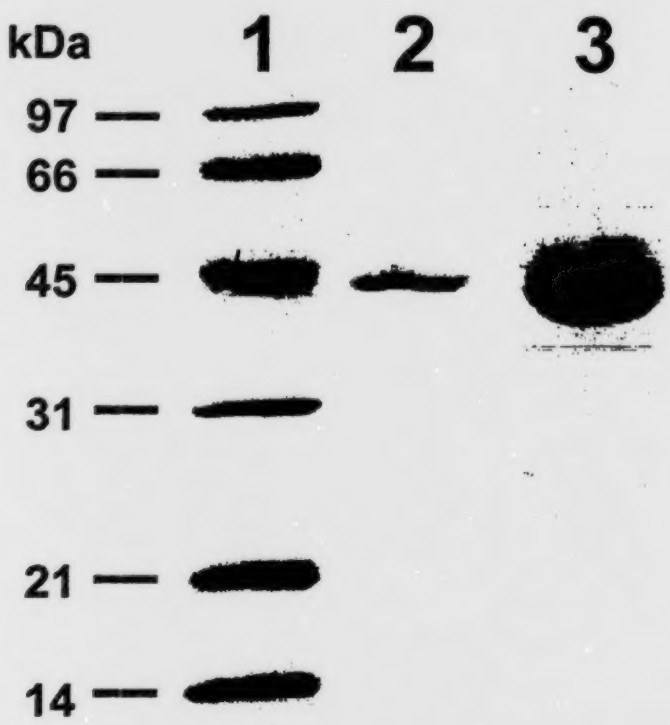



Figure 2. SDS-PAGE analysis of purified *B. pseudomallei* 319a flagellin protein. Proteins were denatured in sample buffer, electrophoresed on a 4% stacking and 12.5% separating polyacrylamide mini gel apparatus and stained with CBB R-250. Lane 1, standard protein markers; lane 2, flagellin protein (5 μg); lane 3, flagellin protein (50 μg).

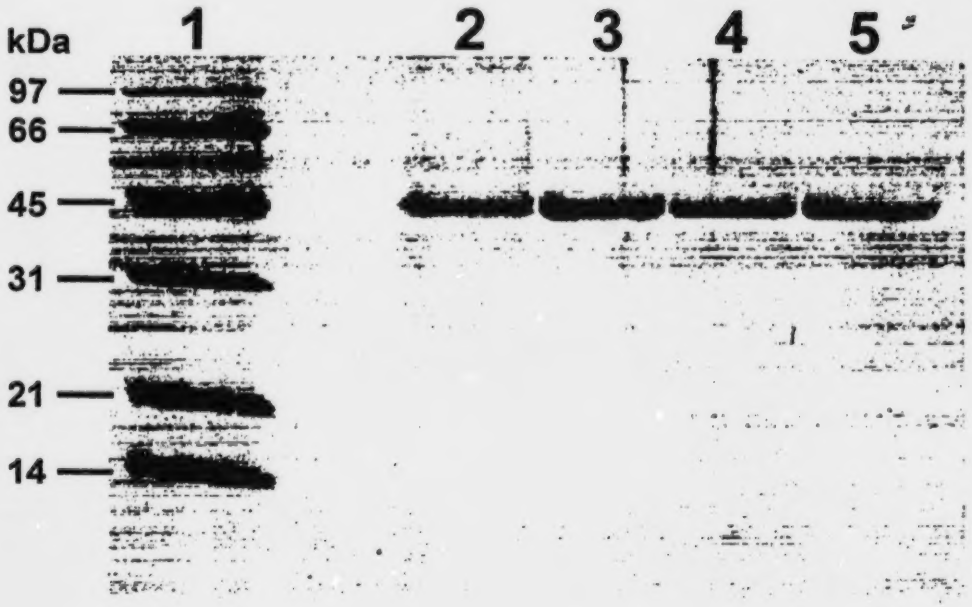



Figure 3. SDS-PAGE analysis of purified flagellin proteins from four different strains of *B. pseudomallei*. Proteins were denatured in sample buffer, electrophoresed on a 4% stacking and 12.5% separating polyacrylamide mini gel apparatus and stained with CBB R-250. Lane 1, standard protein markers; lane 2, 307d flagellin protein (5 μg); lane 3, 316c flagellin protein (5 μg); lane 4, 319a flagellin protein (5 μg); lane 5, 1026b flagellin protein (5 μg).

cultures harvested at a mid to late log phase. Typical yields of protein were in the range of 15-20 mg per 5.0 L of cell culture.

Interestingly, when concentrated HCl was used in place of citric acid to depolymerize filaments prior to ultracentrifugation, the protein samples purified with HCl demonstrated the presence of a single protein band migrating with an apparent $M_r$ = 39,000 (Fig. 4). This phenomenon could also be reproduced if citric acid purified flagella were incubated for a short period of time in solutions in which the pH had been decreased with HCl. This served to confirm that these lower molecular weight species were most likely degradation products of the mature flagellin monomers.

## 2. Biochemical analysis

Amino acid composition analysis of the purified 319a flagellin protein revealed the presence of Ala, Arg, Asx, Glx, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr and Val residues while Cys and Trp went undetected during the sample analysis. With the 394 amino acid residues detected, an $M_r$ = 40,100 was predicted for the flagellin protein. This data is comparable to the amino acid composition calculated from the cloned *fliC* gene of *B. pseudomallei* 1026b (Table 3). The $NH_2$ -terminal amino acid sequence of the 319a flagellin protein was acquired for the first 17 amino acids of the molecule and revealed that the protein was identical over this stretch of residues to the $NH_2$ -terminal sequence predicted for the *B. pseudomallei* 1026b flagellin (Table 4).

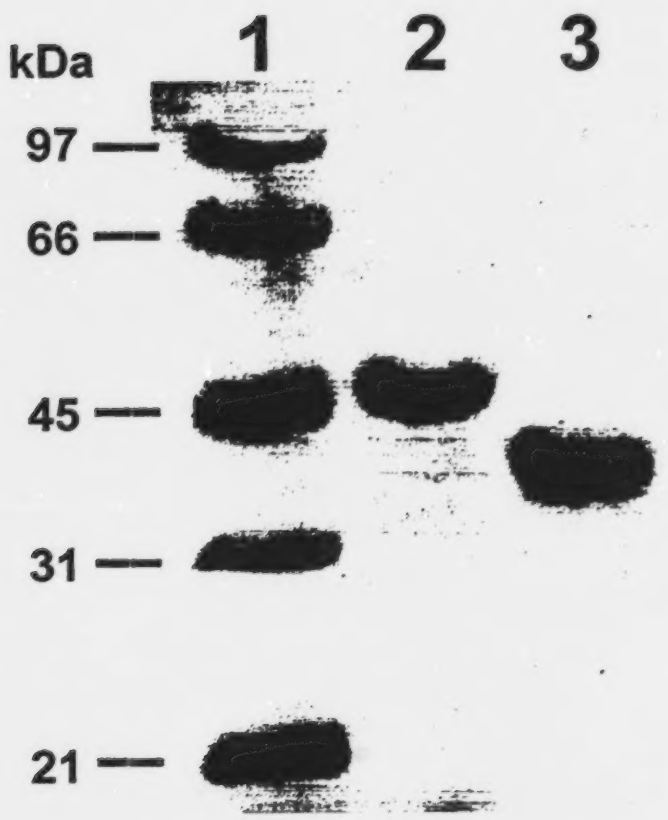



Figure 4-4. SDS-PAGE analysis of *B. pseudomallei* 319a flagellin proteins purified using either 2.0 M citric acid or concentrated HCl during the depolymerization step. Proteins were denatured in sample buffer, electrophoresed on a 4% stacking and 12.5% separating polyacrylamide mini gel apparatus and stained with CBB R-250. Lane 1, standard protein markers; lane 2, 319a flagellin purified using 2.0 M citric acid ( 5 μg ); lane 3, 319a flagellin purified using concentrated HCl ( 5 μg ).

Table 3. Comparison of the amino acid compositions of flagellin proteins from four *B. pseudomallei* strains.

| Amino acid | Number of amino acid residues per flagellin protein | | | |
|---|---|---|---|---|
| | *B. pseudomallei* [a] ATCC 15682 ( FliC ) | *B. pseudomallei* [a] ATCC 23343 ( FliC ) | *B. pseudomallei* [a] 1026b ( FliC ) | *B. pseudomallei* [b] 319a ( FliC ) |
| Ala | 59 | 59 | 59 | 58 |
| Arg | 8 | 8 | 8 | 9 |
| Asp | 13 | 13 | 13 | |
| Asn | 32 | 32 | 32 | 47 ( Asx ) |
| Cys | 0 | 0 | 0 | ND [c] |
| Glu | 4 | 4 | 4 | |
| Gln | 47 | 47 | 47 | 53 ( Glx ) |
| Gly | 33 | 33 | 33 | 36 |
| His | 0 | 0 | 0 | 2 |
| Ile | 21 | 21 | 21 | 24 |
| Leu | 34 | 34 | 34 | 34 |
| Lys | 7 | 7 | 7 | 9 |
| Met | 6 | 6 | 6 | 3 |
| Phe | 7 | 7 | 7 | 8 |
| Pro | 4 | 4 | 4 | 6 |
| Ser | 49 | 49 | 49 | 43 |
| Thr | 37 | 37 | 37 | 35 |
| Trp | 1 | 1 | 1 | ND [c] |
| Tyr | 2 | 2 | 2 | 3 |
| Val | 24 | 24 | 24 | 24 |

[a] Amino acid compositions are based upon the primary sequences predicted from cloned flagellin genes.

[b] Amino acid composition based upon biochemical analysis of purified protein.

[c] ND; not detected.

Table 4. Comparison of the $NH_2$ -terminal amino acid sequences of flagellin proteins from four *B. pseudomallei* strains.

| *B. pseudomallei* strain | Sequence |
|---|---|
| 319a [a] | $^{1}$MLGINSNINSLVAQQNL$^{17}$ |
| 1026b [b] | $^{1}$MLGINSNINSLVAQQNL$^{17}$ |
| ATCC 15682 [b] | $^{1}$MLGINSNINSLVAQQNL$^{17}$ |
| ATCC 23343 [b] | $^{1}$MLGINSNINSLVAQQNL$^{17}$ |

[a] $NH_2$ -terminal sequence is based upon the biochemical analysis of purified protein.

[b] $NH_2$ -terminal sequences are based upon the primary sequences predicted from the cloned flagellin genes.

Analysis of the proteins encoded by the *fliC* gene sequences of *B. pseudomallei* strains ATCC 15682 and ATCC 23343 (GenBank accession numbers U82287 and U82286) demonstrated 100% identity to the primary sequence predicted for that of *B. pseudomallei* 1026b (Table 3).

### 3. Immunochemical analysis

Rabbit polyclonal antiserum raised against the purified 319a flagellin protein was shown, via Western blotting, to react with purified 319a flagellin as well as the flagellins expressed by a variety of *B. pseudomallei* strains. The various flagellin proteins appear as immunostained bands with an $M_r$ = 43,000 (Fig. 5). The MAPS II-purified IgG fraction of the polyclonal serum reacted similarly with the same flagellin molecules (data not shown). Immuno-dot blot analysis using both the polyclonal serum and MAPS II IgG fraction demonstrated the reactivity of 64/64 *B. pseudomallei* strains with both of the antiflagellin antibody preparations. The negative control *P. aeruginosa* DG1 was unreactive with either the serum or purified antibodies (data not shown).

### 4. Motility inhibition assays

To determine the functional activity of the 319a antiflagellin polyclonal serum, we examined the ability of the serum to inhibit the motility of

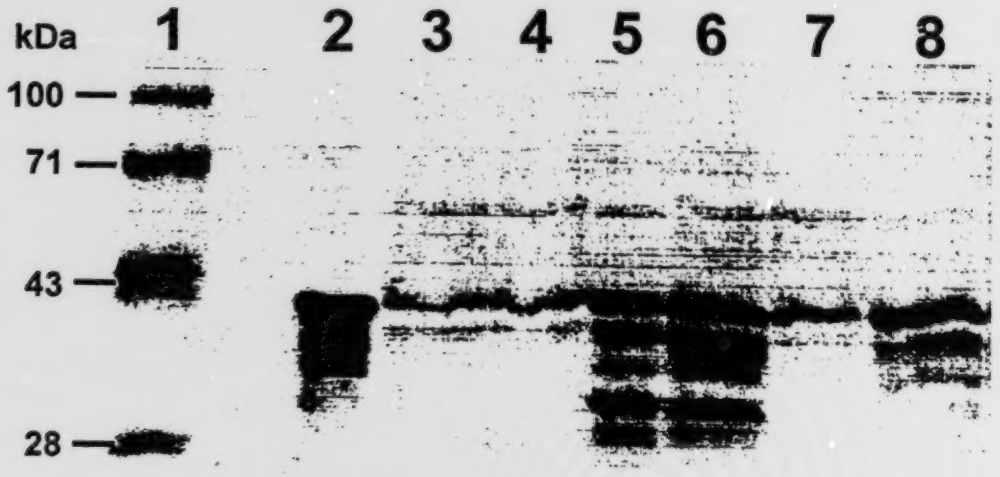



Figure 5. Western immunoblot analysis of whole cell extracts reacted with antiserum raised against *B. pseudomallei* 319a flagellin (1:1,000 dilution). Whole cells were lysed with sample buffer, boiled for 5 minutes and loaded in equal amounts to the wells of a 4% stacking and 10% separating standard polyacrylamide gel apparatus. Lane 1, prestained protein molecular weight markers (phosphorylase b [100.5 kDa], bovine serum albumin [71.8 kDa], ovalbumin [43.2 kDa], carbonic anhydrase [28.5 kDa]: Bethesda Research Laboratories ); lane 2, purified 319a flagellin (2 μg); lane 3, 1026b whole cell extract; lane 4, 415d whole cell extract; lane 5, 319a whole cell extract; lane 6, 316c whole cell extract; lane 7, 307d whole cell extract; lane 8, 304f whole cell extract.

*B. pseudomallei* 319a in a soft agar assay. Specificity was examined by testing the ability of the antiserum to inhibit motility of *P. aeruginosa* DG1. Anti-319a flagellin polyclonal serum at a dilution of 1:1000 or less inhibited the motility of the *B. pseudomallei* strain but was unsuccessful at preventing the motility of the *P. aeruginosa* strain (Fig. 6).

**5. Passive immunization studies.**

Diabetic infant rats were inoculated with varying amounts of *B. pseudomallei* 316c and administered i.p. either 1.5 mg of anti-319a flagellin IgG or a PBS solution (control). After the sixth day post inoculation only 4/20 of the rats in the control groups were still alive, whereas in the immunized groups 11/20 had survived the same period. The groups demonstrating the most significant differences between one another were the $10^4$ inoculum groups. Four of five immunized rats had survived the bacterial challenge in comparison to zero of the five rats in the control group. The calculated $LD_{50}$ for the control group was $<10^3$, while that for the immunized group was $1.0 \times 10^5$ (Table 5 ). In a separate experiment, the PBS controls and the anti-319a antibodies were introduced i.v. into the tail veins of the rats. In PBS treated controls, 16/20 rats had died by day six, whereas the immunized animals displayed a mortality rate of only 4/20 over the six days. In contrast to the previous study, animals demonstrated a more pronounced survival rate when the IgG was

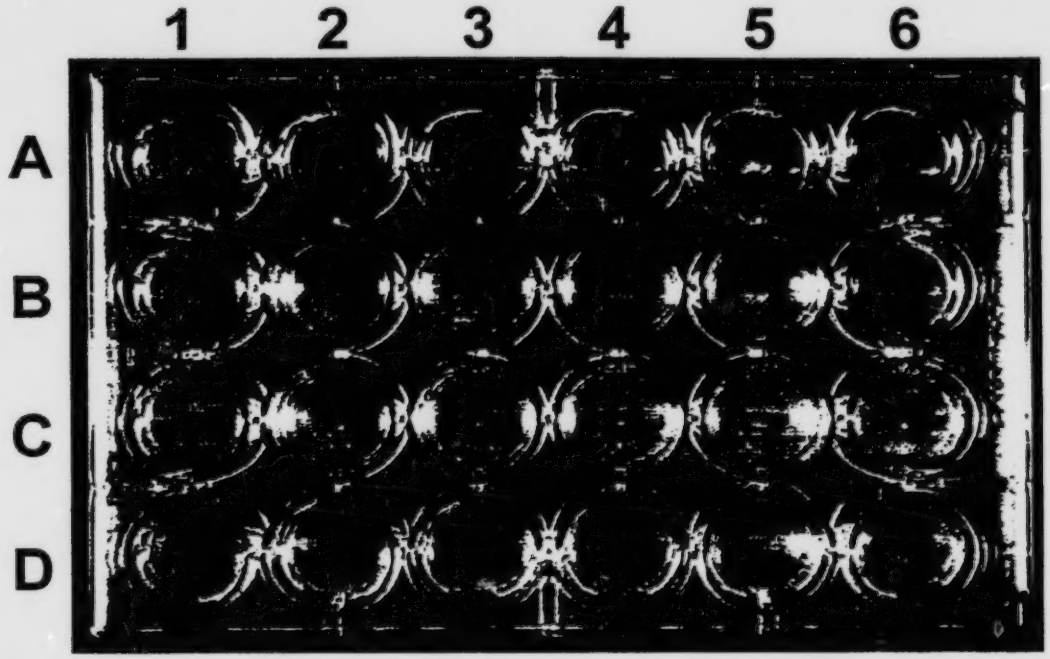



Figure 6. Motility inhibition assessment of *B. pseudomallei* 319a and *P. aeruginosa* DG1 reacted with varying dilutions of antiserum raised against 319a flagellin. The wells in rows A and B were inoculated with *B. pseudomallei* 319a. The wells in rows C and D were inoculated with *P. aeruginosa* DG1. Anti-319a flagellin antiserum (rows A and C) or Type II LPS specific MAb (rows B and D) was added to the wells. Wells: 1, PBS control; 2, 1:5,000 dilution; 3, 1:1,000 dilution; 4, 1:500 dilution; 5, 1:100 dilution; 6, 1:10 dilution.

Table 5. Passive immunoprotection by anti-319a flagellin IgG in diabetic rats challenged with a heterologous *B. pseudomallei* strain.

| Inoculum size ( CFU ) | Mortality ( n=5 ) | | | |
|---|---|---|---|---|
| | Experiment A [a] | | Experiment B [b] | |
| | Control ( BSA i.p. ) | Immunized ( IgG i.p. ) | Control ( BSA i.v. ) | Immunized ( IgG i.v. ) |
| $10^3$ | 4 | 3 | 3 | 2 |
| $10^4$ | 5 | 1 | 5 | 0 |
| $10^5$ | 3 | 1 | 4 | 1 |
| $10^6$ | 4 | 4 | 4 | 1 |

[a] $LD_{50}$ = < $10^3$ control; $LD_{50}$ = $10^5$ immunized

[b] $LD_{50}$ = < $10^3$ control; $LD_{50}$ = > $10^6$ immunized

administered i.v. rather than i.p. The $10^4$, $10^5$ and $10^6$ inoculum groups displayed a much lower mortality rate when protected with antibodies. The calculated $LD_{50}$s for this control group was $<10^3$, while that for the immunized group was $>10^6$ (Table 5).

At the completion of the experiments, blood samples obtained from the surviving animals by sterile cardiac puncture were plated onto tryptic soy agar (TSA) and incubated overnight at 37°C. All of the unimmunized animals were found to be bacteremic due to the presence of heavy bacterial growth on the TSA plates, while immunized animals demonstrated no signs of bacteremia.

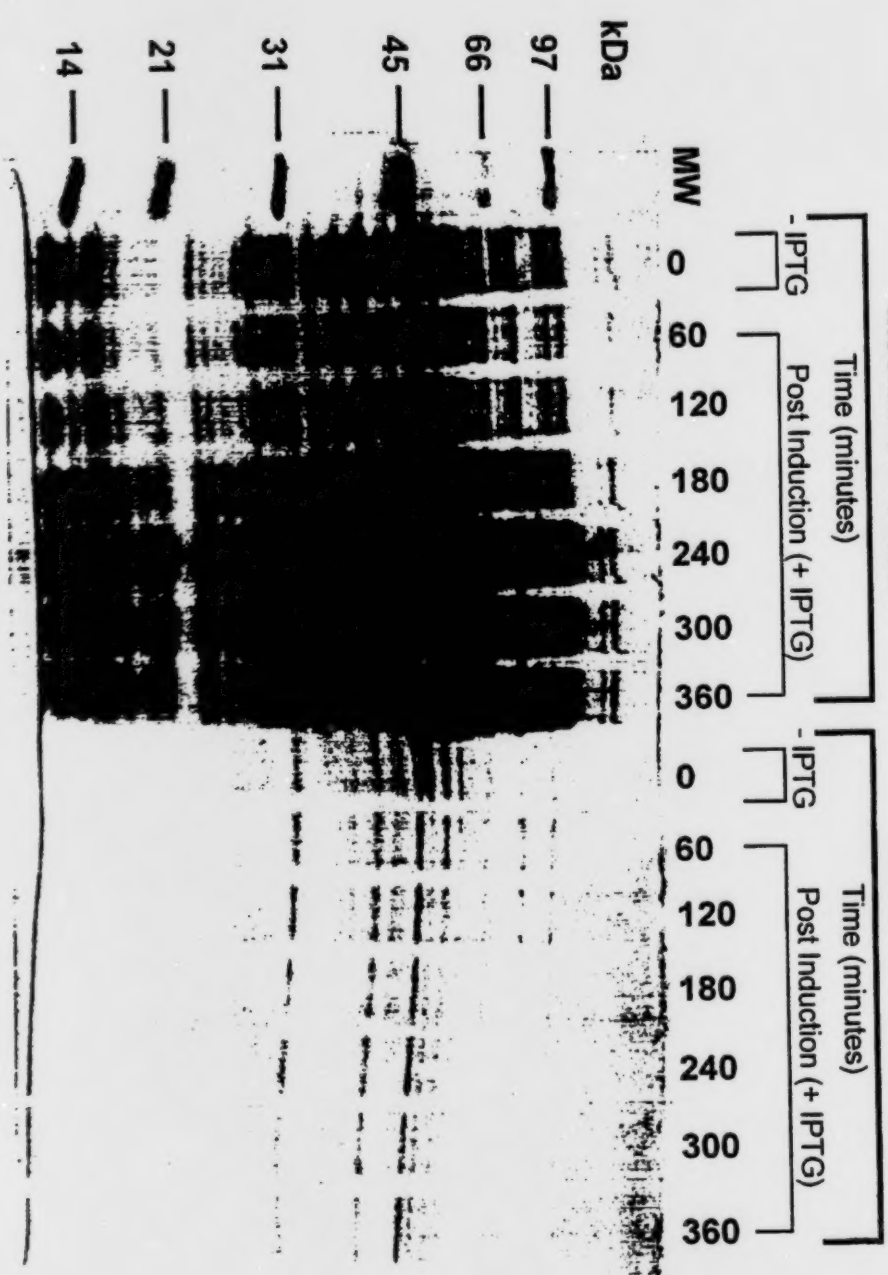
## 6. Construction, expression and purification of rFliC

In order to express a rFliC molecule in an *E. coli* background, the *B. pseudomallei* 1026b *fliC* locus was PCR amplified from the pPBS2H template and ligated into the pTrcHis C expression vector. Appropriate restriction endonuclease sites were incorporated into the ends of the oligonucleotide PCR primers such that the amplified product could be directly cloned in frame with the histidine tag encoding sequence of the pTrcHis C expression vector. The construction of this tightly regulated expression vector facilitated the synthesis of a fusion protein that could be readily purified from cellular contaminants via the exploitation of the metal ion binding moiety incorporated into the $NH_2$ -terminus of the recombinant protein.

In order to standardize conditions for maximal expression of the rFliC, a time course of flagellin expression by isopropylthio-β-D-galactoside (IPTG) induction was carried out at 37°C. As shown in Fig. 7, optimal expression of the fusion protein was reached approximately 4-5 hours post induction with the appearance of a 46 kDa band. By up scaling the protein expression system, recombinant protein was isolated under denaturing conditions and purified via nickel chelate chromatography at quantities of 3 mg per 50 ml of culture medium (see Materials and Methods). The vast majority of the product partitioned into the insoluble fraction of the whole cell extracts suggesting that the accumulating protein molecules were probably harvested in the form of inclusion bodies.

SDS-PAGE analysis of column purified samples demonstrated the presence of the recombinant flagellin (apparent $M_r$ = 46,000) along with some minor contaminating protein bands (Fig. 8). The difference in the relative mobility observed between the protein expressed in *E. coli* DH5α and the *B. pseudomallei* 319a flagellin is likely due to the presence of the fusion peptide incorporated into the $NH_2$ -terminal domain of the recombinant protein. Western immunoblot analysis of the rFliC confirmed that the protein was also capable of reacting with the anti-319a flagellin antiserum (Fig. 9).

Figure 7. SDS-PAGE analysis of the expression of recombinant flagellin protein as a function of time of induction. Induction of recombinant FliC was conducted at 37°C for 1-6 hours following the addition of 1 mM IPTG to a mid log phase culture of *E. coli* DH5α (pTHF-CK/E) as described in Materials and Methods. Aliquots were removed from the cell culture at 1 hour intervals post induction. Soluble and insoluble whole cell fractions were mixed with sample buffer, electrophoresed on a 4% stacking and 10% separating standard polyacrylamide gel apparatus and stained with CBB R-250. Lane 1, standard protein markers; lanes 2-7, insoluble fractions from cell pellet extracts; lanes 8-12, soluble fractions from the cell pellet extracts.


Insoluble Fractions
Soluble Fractions
Time (minutes)
Post Induction (+ IPTG)
- IPTG
MW
kDa
0
60
120
180
240
300
360
97
66
45
31
21
14

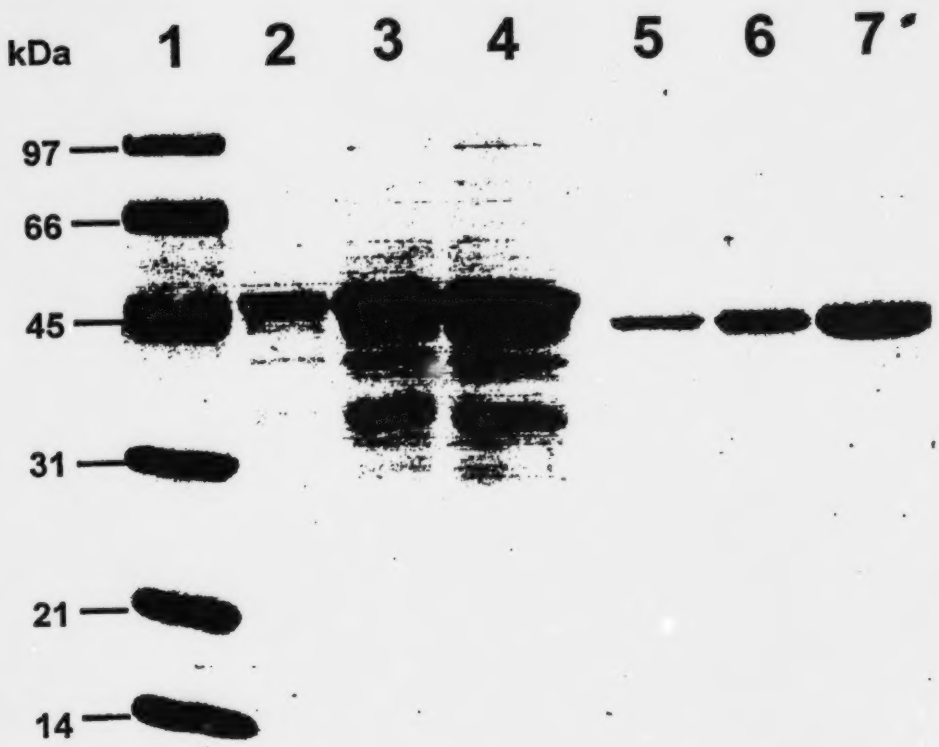



Figure 8. SDS-PAGE analysis of purified *B. pseudomallei* 319a flagellin as compared to purified rFliC. Proteins were denatured in sample buffer, electrophoresed on a 4% stacking and 12.5% separating polyacrylamide mini gel apparatus and stained with CBB R-250. Lane 1, standard protein markers; lanes 2-4, 5 μg, 10 μg and 20 μg recombinant flagellin respectively; lanes 5-7, 2 μg, 5 μg and 10 μg purified 319a flagellin respectively.

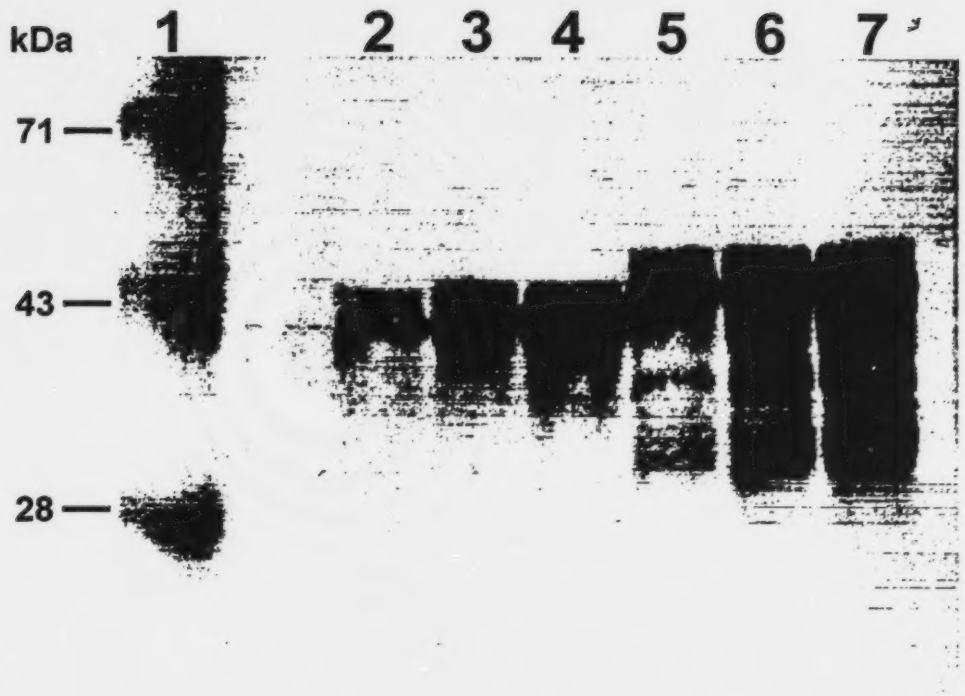



Figure 9. Western immunoblot analysis of purified *B. pseudomallei* 319a flagellin and purified rFliC with 319a antiflagellin antiserum (1:1,000). Lane 1, prestained protein molecular weight markers; lanes 2-4, 1 μg, 2 μg and 5 μg of purified 319a flagellin respectively; lanes 5-7, 1 μg, 2 μg and 5 μg of recombinant flagellin respectively.

## D. DISCUSSION

In this study, the flagellar filaments from *B. pseudomallei* strains 307d, 316c, 319a and 1026b were isolated and purified to homogeneity. By conducting a series of initial small scale isolations, it was determined that the highest yield of protein could be obtained when mid to late log phase cultures were harvested. This result was consistent with previous studies establishing that the maximal rate of *fliC* transcription occurred during this phase of growth (19). Analysis of the purified protein samples by SDS-PAGE demonstrated that filaments synthesized by this organism are the result of the polymerization of a single 43,400 Da flagellin species. The presence of the single subunit in the flagellar filaments of *B. pseudomallei* strains is consistent with evidence suggesting that non-sheathed bacterial flagella display only a single species of protein when disaggregated (44). An exception to this rule, however, appears to be exhibited by *Bacillus pumilis* 101 in which two immunologically distinct monomers co-polymerize to form the filament structure (61).

Of the virulent clinical isolates analyzed to date, there appears to be a significant degree of size homology amongst flagellins synthesized by *B. pseudomallei* strains. While this feature should prove to be immunologically beneficial for the construction of a vaccine candidate, further studies will be required to determine to what extent this protein homology exists. In contrast to *B. pseudomallei* strains, flagellin proteins expressed by *P. aeruginosa* (3) and *E. coli* (69) strains demonstrate a high degree of heterogeneity with regards to subunit size. A hypothesis which explains the advantage of this flagellin size diversity suggests that by modifying protein structure at a genetic level, via the addition or deletion of regions within the variable central domain, the antigenicity of the molecules can be modulated (82). These spontaneous

genetic rearrangements in turn result in the evolution of a flagellin antigens that may confer a selective advantage on the organism in its surroundings (10, 59).

While attempting to define a protocol for purifying *B. pseudomallei* flagellins, a curious observation was made regarding the specific effects of acids on the outcome of the protein samples. When citric acid was used to depolymerize flagellar filaments, a single protein species with an $M_r$ = 43,400 was isolated. In contrast, samples purified with HCl demonstrated the presence of a single protein band, but with an $M_r$ approximately 4,000 Da smaller in comparison. One explanation that may account for this anomaly is that *B. pseudomallei* flagellins in the presence of HCl (which has a much higher dissociation constant than citric acid) undergo acid catalyzed hydrolytic cleavage at the terminal ends of the protein resulting in the smaller molecular weight species. Alternatively, HCl may be facilitating the removal of acid labile posttranslational modifications from the protein subunits. Since the predicted mass of *B. pseudomallei* flagellins as determined from cloned gene sequences is approximately 39.1 kDa, the latter hypothesis seems to be worth exploring in further detail.

It has been demonstrated with a number of flagellin species that when a predicted $M_r$ from gene sequence analysis does not correlate with the apparent $M_r$ obtained by SDS-PAGE, the cause can often be attributed to the presence of posttranslational modifications (Table 5). This is exemplified in the flagellins expressed by *P. aeruginosa* strains were phosphorylation of tyrosine residues in both Type a and Type b proteins have been documented (46, 47). Similar results have also shown that the glycosylation of *C. jejuni* flagellin proteins can account for the size

Table 5. Characterization of flagellin proteins from six bacterial species.

| Organism / flagellin subunit | Total number of residues/ molecule [a] | $M_r$ ( $10^3$ ) | | Posttranslational modifications |
|---|---|---|---|---|
| | | Predicted [a] | Apparent [b] | |
| *B. bronchiseptica* GP1SN ( FlaA ) | 391 | 40.5 | 40.0 | none |
| *B. pseudomallei* 1026b ( FliC ) | 388 | 39.3 | 43.4 | ? |
| *C. coli* VC167 ( FlaB ) | 573 | 59.2 | 61.5 | glycosylation |
| *L. pneumophila* serogroup 1 ( FlaA ) | 475 | 48.0 | 47.0 - 48.0 | none |
| *P. aeruginosa* PAK ( Type a ) | 394 | 40.0 | 43.0 - 45.0 | tyrosine phosphorylation |
| *P. mirabilis* BB2000 ( FliC1 ) | 365 | 39.1 | 39.8 | none |

[a] Calculated from primary amino acid sequences derived from cloned genes (2, 6, 31, 36, 78).

[b] Based upon SDS-PAGE analysis of purified proteins.

discrepancies observed between the mass predictions from gene sequence analyses and those obtained from SDS-PAGE (23). While the role of posttranslational modifications are currently under investigation, there is strong evidence to suggest that some play a role in eliciting protective immunity (30).

Protein sequence analysis of the *B. pseudomallei* 1026b flagellin, as predicted from the cloned *fliC* gene, revealed that the amino acid content and regions of the primary structure are consistent with the residue content and primary sequences defined for other characterized flagellin proteins. The similarity is particularly obvious when residue compositions are compared since flagellins typically lack cysteine and histidine residues and are generally low in basic and aromatic amino acids (83). The data acquired for the *B. pseudomallei* 319a protein itself was shown to be compatible with the 1026b flagellin. While inconsistencies observed between the two flagellin proteins will only be rectified if the 319a *fliC* gene is cloned and sequenced, the biochemical analysis of the purified 319a protein served to confirm that the 43,400 kDa proteins isolated in this study were indeed flagellins molecules. Sequence analysis of the *B. pseudomallei* ATCC 15682 and ATCC 23343 flagellin proteins also supports mounting evidence suggesting that a high degree of homology exists amongst flagellins expressed by *B. pseudomallei* strains.

Western blot analysis of the purified flagellins, as well as whole cell extracts from a number of *B. pseudomallei* strains, confirmed that the flagellin antigens are both structurally and immunologically conserved throughout this bacterial species. Immuno-dot blot analysis of *B. pseudomallei* clinical isolates

with the 319a polyclonal antiserum also demonstrated that 64/64 of strains were reactive with both the whole serum and the purified IgG fraction. The presence of cross-reacting epitopes exhibited by these 64 strains again suggests that flagellins expressed by *B. pseudomallei* strains are immunologically and structurally similar. This result correlates well with those derived via the SDS-PAGE and protein sequence analyses conducted throughout this study. In order to confirm, however, the putative existence of a serotypically conserved flagellin antigen, we will need to conduct future immunological analyses with monoclonal antibodies.

Polyclonal antiserum raised against the homogeneous 319a flagella preparation was shown to inhibit cell motility of *B. pseudomallei* 319a, but not *P. aeruginosa* DG1 *in vitro*, confirming that the 319a antiflagellin antiserum is both functional and specific. While the exact mechanism of how antiflagellin antibodies mediate an inhibitory effect on motility is not fully understood, previous studies have demonstrated that immunoglobulin interactions at the surface of the bacterial flagella tend to disrupt the rotational action of the flagellar filaments leading to the inability of the microbe to propel itself effectively (58). Since mobility has been demonstrated to be an important virulence trait associated with many bacterial pathogens (5, 58, 63), disruption of this activity with immobilizing antibodies *in vivo* may prove to be an advantageous prophylactic measure against infections.

In this study, we have demonstrated that passive immunization of diabetic infant rats with immunoglobulins raised against purified the 319a

flagella preparation protected the animals from a *B. pseudomallei* challenge. While the mechanism(s) of this protective effect are still under investigation, the interaction of the immobilizing and perhaps also opsonizing antibodies with flagellar filaments may disrupt the normal function of the flagella resulting attenuated pathogenicity as well as aid in the phagocytosis of this organism (63). Further studies, however, are required to confirm these theories.

Throughout these studies, we have been successful in demonstrating that flagellin proteins expressed by *B. pseudomallei* strains should be characterized in finer detail due to their potential as vaccine candidates for prophylaxis against melioidosis. In order to accomplish this, however, it would be desirable to purify these proteins on a larger scale. Unfortunately, since *B. pseudomallei* is particularly virulent and could potentially pose a significant biohazard risk to laboratory personnel, the scaling up of bacterial cultures to many hundreds of litres in a Bio-reactor is neither practical nor appealing. In order to address this issue, we have constructed a recombinant protein expression system enabling us to express and purify a rFliC molecule in an *E. coli* background. This was accomplished by cloning a PCR amplified *B. pseudomallei* 1026b *fliC* gene into the prokaryotic expression pTrcHis C. Since the analysis of the fusion protein has demonstrated that it is both structurally and immunologically homologous to flagellins purified from *B. pseudomallei* strains, large scale purification of the rFliC should no doubt prove to be beneficial for assessing the immunoprotective capacities of *B. pseudomallei* flagellins in the future. As SDS-PAGE analysis of the rFliC

preparation demonstrates, however, a modification in the purification procedure will be required in order to achieve a higher quality protein sample.

# Construction of Conjugate Vaccine Candidates for Active Immunization Against Melioidosis

## A. INTRODUCTION

*Burkholderia pseudomallei* is the causative agent of melioidosis, a disease that afflicts both humans and animals (13, 52, 86). The manifestations of melioidosis are commonly represented by acute, sub-acute and chronic illnesses, with the clinical indications of some stages often being mistaken for those associated with malaria, plague, pneumonia and miliary tuberculosis (16, 52, 73).

In previous studies, it has been demonstrated that polyclonal and monoclonal antisera raised against *B. pseudomallei* flagellin proteins (9), lipopolysaccharides (LPS), and a tetanus toxoid glycoconjugate molecule (11) provided passive protection against *B. pseudomallei* infections in animal model experiments (9, 11). It is clear, however, that an active vaccine preparation would be of most benefit to individuals at risk of acquiring *B. pseudomallei* infections. As spectroscopic and serological analysis of *B. pseudomallei* flagellin proteins and O-polysaccharides (PS) have also demonstrated that a high degree of conservation in these molecules, both on a structural and immunological basis, is present throughout this bacterial species (9, 11, 48, 64), we have hypothesized that a conjugate molecule incorporating both flagellin protein and PS antigens would be a reasonable vaccine candidate for use in active immunization against melioidodsis.

It has been well documented that T cell-independent type 2 (TI-2) antigens such as bacterial polysaccharides are capable of eliciting

protective antibody responses in adults (20, 41), but act poorly as immunogens in the elderly, in immunocompromised populations and in children less than 18 months of age (20, 29, 40, 41, 67). In adult populations, TI-2 antigens typically stimulate the synthesis of antigen specific immunoglobulin M (IgM), but often fail to evoke augmented immune responses and isotype conversions following boosting with carbohydrate preparations. This phenomenon is primarily due to the inability of activated B lymphocytes to recruit $CD4^+$ T cell (Th) involvement via major histocompatibility complex type II (MHC II) restricted events (37, 57). In neonates, extremely poor responses to TI-2 antigens are also MHC II related, however, the problems are compounded by insufficient natural killer cell (NK) cytokine stimulation and the presence of defective B cell membrane Ig (mIg) signaling pathways (7, 20, 57, 75). In order to remedy this situation, a number of bacterial carbohydrates (such as capsular polysaccharides or somatic O-antigens) have been covalently coupled to protein or peptide carrier molecules in order to facilitate T cell-dependent (TD) immune responses against the particular carbohydrate moieties (14, 28, 39, 49, 53, 80, 84). The presence of T cell epitopes inherent to peptide or proteinacious carriers is believed to be facilitate these events (7).

A flagellin-polysaccharide conjugate molecule would possess a variety of desirable attributes that need be considered in the rational design of a conjugate vaccine. The incorporation of two protective antigens from the same organism should enhance the immunological repertoire of the vaccine recipient, while at the same time affording protection against strains that

display serotypically distinct antigens of one of the two components. The conjugation of the PS to the carrier protein should also elicit an augmented immune response against the PS component while at the same time evoking desirable immunoglobulin (Ig) class switching events. Most importantly, the use of an active vaccine containing the PS portion of LPS but not the toxic component of LPS, namely lipid A, conjugated to flagellin protein would obviate the toxic side effects of LPS but take advantage of the protective response to the PS moiety.

In this study, we report the synthesis of a number of PS-flagellin conjugate molecules and the preliminary assessment of the immunological characteristics of such constructs for potential use in immunoprophylaxis against *B. pseudomallei* infections.

## B. MATERIALS AND METHODS

### 1. Bacterial strains, media and growth conditions

The bacterial strains used in this study are described in Table 1. All stock cultures were maintained in either 10% skim milk or 20% glycerol suspensions and stored at -70°C. *B. pseudomallei* strains were grown at 37°C in Luria-Bertani (LB) broth or on LB agar plates (Difco Laboratories Inc.). For animal studies, *B. pseudomallei* cultures were grown at 37°C in TSBDC media (8).

### 2. Isolation and purification of conjugate antigens.

Flagellar filaments were obtained by previously described methods (9). *B. pseudomallei* 319a cells, retained from the flagellar filament isolations, were enzymatically treated as previously described by Johnson *et al* (43) prior to extraction with hot phenol as outlined by Westphal *et al* (81) in order to obtain crude LPS extracts. The resulting phenol-water mixture was dialyzed against running tap water until phenol odor was undetectable and then clarified by centrifugation at 7,000 x g for 15 minutes prior to lyophilization. The fluffy white solid was re-solubilized to 30 mg/ml in 10 mM Tris, 1 mM calcium chloride and 1 mM magnesium chloride (pH 7.4) and incubated for 2 hours at 37°C with 10 μg/ml DNase and 20 μg/ml RNase, followed by incubation with 50 μg/ml

Proteinase K at 45°C for 6 hours. The supernatant was ultracentrifuged at 80,000 x g for 1 hour at 4°C, and the gel like substance at the bottom of the centrifuge tubes was removed and dialyzed against distilled water prior to lyophilization. The resulting purified LPS was shown to contain < 1% nucleic acid or protein on a dry weight basis.

Polysaccharide was obtained by solubilizing LPS in 3% acetic acid at 10 mg/ml, and heating to 100°C in a sealed ampoule for 2 hours (24). Insoluble lipid A moieties liberated during the acid hydrolysis were removed by centrifugation at 4,000 x g for 15 minutes, and the supernatant was dialyzed against distilled water followed by lyophilization. The PS was further purified on a Sephadex G-50 (Pharmacia Biotech) column (2.5 x 40 cm) using 100 mM sodium phosphate buffer (pH 7.4) as the eluting solvent. Fractions that emerged with a $K_{av} < 0.45$ ($K_{av} = V_e - V_o/V_t - V_o$) and which were positive for carbohydrate were pooled, dialyzed extensively against distilled water and lyophilized.

### 3. Conjugate synthesis.

The most immunogenic conjugate of those constructed in this study was synthesized by modification of methods previously described (11, 15). Briefly, a 40 mg sample of PS was dissolved in 8 ml of distilled water, cooled to 4°C, and adjusted to pH 10.5 with 0.10 M NaOH. A 40 µl aliquot of a 100 mg/ml solution of cyanogen bromide (CNBr; Sigma Chemical Co.) in acetonitrile was

added to the PS and allowed to react for 10 minutes. The pH of the reaction was reduced to 8.5 with 0.50 M $NaHCO_3$ (Sigma Chemical Co.) followed by the introduction of 160 mg of adipic acid dihydrazide (ADH; Sigma Chemical Co.), and the mixture was allowed to stir overnight at 4°C. The derivatized PS at this point was extensively dialyzed against distilled water and lyophilized. This product was termed PS-ADH.

A solution of 20 mg PS-ADH in 5 ml of distilled water was acidified to pH 4.3 with 0.10 M HCl, and to this was added 2 ml of distilled water containing 2 mg of flagellin protein and 200 mg of 1-ethyl-3-(3-diethylaminopropyl) carbodiimide (Sigma Chemical Co.). The reaction mixture was stirred for 4 hours, following which the product of the reaction was dialyzed against distilled water, clarified by low speed centrifugation and concentrated by ultrafiltration to approximately 3 ml. The conjugate mixture was applied to a Sepharose CL-4B gel column (2.5 x 40 cm) and eluted with a 100 mM sodium phosphate buffer (pH 7.4). Fractions demonstrating both protein and carbohydrate content were pooled, dialyzed against distilled water and lyophilized. This glycoconjugate was named PS-ADH-FLA.

Coupling of PS to flagellin protein was also accomplished by activating the PS with either a combined reduction/oxidation procedure (42), or a simple one step oxidation processes (17). Following conjugation to protein by reductive amination, these glycoconjugates were termed (red/ox)PS-FLA and (ox)PS-FLA respectively.

## 4. Analytical methods.

Protein content was determined by the bicinchoninic acid (BCA) protein assay method (Pierce Chemical Co.), and the presence of carbohydrate was assessed by the phenol-sulphuric acid method (25). Standard curves generated with purified flagellin protein or PS preparations were used to quantitate the percentage of each component in the various glycoconjugates. Nucleic acid content was estimated from absorbance 260 nm (Abs 260 nm) readings of a 1 mg/ml solution of LPS. The average relative mobility ($M_r$) of the PS-ADH-FLA conjugate was calculated from a linear regression analysis of data obtained by calibrating a Sephacryl S-300 HR (Pharmacia Biotech) gel column (2.5 x 40 cm) with high molecular weight globular protein standards (Pharmacia Biotech) eluted with 100 mM sodium phosphate buffer (pH 7.4).

## 5. Antibody production.

Antibodies to the various glycoconjugates were prepared by administration of Freund's complete adjuvant (FCA; Bethesda Research Laboratories) emulsified with filter sterilized preparations of the individual conjugates in 10 mM phosphate buffered saline pH 7.0 (PBS). The rabbits (New Zealand White, 2 to 2.5 kg) each received an intramuscular injection of 0.50 ml of the emulsion in both their right and left hind thigh muscles. Each injection contained 100 μg of conjugate per ml. The rabbits were thereafter

boosted on days 14 and 28 post primary immunization with Freund's incomplete adjuvant (FIA; BRL) as the emulsifying agent, and test bleeds were taken at these points. Animals were exsanguinated by cardiac puncture under anesthesia at day 42, and serum samples were collected and stored at -70° C until required for use.

**6. Enzyme-linked immunosorbent assays (ELISA).**

Round bottom microtitre plates were coated with 1 μg of either 319a flagellin protein, LPS or PS solubilized in 100 μL of 0.05 M carbonate buffer pH 9.6 and incubated for 2 hours at 37° C. The wells were then washed with PBS plus 0.05% Tween-20 (Aldrich Chemical Co.) and blocked with a 3% solution of skim milk in PBS-Tween for 2 hours at 37° C. The wells were washed, and 100 μl of the immune serum dilutions were added to the appropriate wells followed by incubation at 37°C for 2 hours. Plates were then washed, and a 1/1000 dilution of a goat anti-rabbit IgG-horse radish peroxidase (HRP) conjugate (Bio-Rad Laboratories) was added to each well. The plates were incubated for 2 hours at 37° C, washed and developed with an ABTS substrate solution (Kirkegaard and Perry Laboratories, Inc.) for 20 minutes and read at Abs 405 nm. The highest dilution of immune serum that reacted against a specific antigen was expressed as the reciprocal value of that dilution.

## 7. Electrophoresis.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 4% stacking and 12% separating (wt/vol) acrylamide slab gel system (51). Samples were solubilized under denaturing conditions and heated to 100°C for 5 minutes prior to being loaded into the gel wells. Electrophoresis of the samples was accomplished at 20 mA until the dye front had reached 1 cm from the bottom of the gel. Electrophoretic transfer of antigens to a nitrocellulose membrane (Bio Rad Laboratories) was performed as described by Towbin *et al* (79) using a Bio Rad Trans-Blot Cell (Bio Rad Laboratories) operating at 50 V for 4 hours at 4°C.

## 8. Immunoblotting.

Following electrophoretic transfer of antigen to nitrocellulose membrane, unbound sites were blocked with a 3% skim milk solution in 20 mM Tris buffered saline pH 7.5 (TBS) overnight at 4°C. The membrane was cut into strips, placed into small staining trays and left to react for one hour at 37°C in a rocking incubator with the primary antibody diluted appropriately in TBS. The strips were then washed thoroughly for 1 hour with TBS and allowed to react with either a 1/1000 dilution of goat anti-rabbit IgG-HRP conjugate (Bio Rad Laboratories) or goat anti-mouse IgM-HRP conjugate (Sigma Chemical Co.). The reaction was allowed to proceed for 1 hour at 37°C with gentle agitation.

The membranes were washed thoroughly again for 1 hour and were developed with an HRP Color Development Reagent (Bio-Rad Laboratories) (12).

**9. Immuno-dot blot assay.**

Stock *B. pseudomallei* clinical isolates were streaked for isolation onto LB agar plates and incubated at 37°C for 36 hours. Individual colonies were picked from the plates, resuspended in 500 µl PBS and boiled for 5 minutes. A 5 µl aliquot of the individual strain suspensions was applied to the nitrocellulose paper and the membrane support was left to air dry. The nitrocellulose was then reacted with PS-ADH-FLA antiserum as previously described for immunoblotting.

**10. Motility Inhibition Assay.**

Four rows (6 wells in each row) from a 24 well microtitre plate were filled with motility agar (0.8% nutrient broth plus 0.3% agar) which had been rehydrated with the addition of 1/100 dilutions of either anti-PS-ADH-FLA polyclonal rabbit serum, anti-319a flagella specific polyclonal rabbit serum, or a Type II LPS specific monoclonal antibody (MAb) (9, 11). The fourth row was rehydrated with a 1/100 dilution of PBS to serve as a controls. *Pseudomonas aeruginosa* PAO and several *B. pseudomallei* strains were then inoculated into the wells in such a way that each of the organisms would be grown in all four

of the differentially reconstituted agar environments. The plate was incubated at 37°C for 12 hours. After incubation, the wells were examined for signs of cell motility demonstrated by a halo originating from the stab center (9). In all, 59 *B. pseudomallei* clinical isolates were assayed with this method.

**11. Isolation IgG from Rabbit Antisera.**

An Affi-Gel® Protein A MAPS® II Kit (Bio-Rad Laboratories) was used to isolate the IgG fraction of the crude rabbit polyclonal antiserum produced against PS-ADH-FLA. The purified immunoglobulin fraction was utilized for passive immunoprotective studies in an animal model of *B. pseudomallei* infection.

**12. Animal Protection Studies.**

Immunoprotection studies were performed in an animal model of *B. pseudomallei* infection as previously described by Woods *et al* (85). Briefly, 100 Sprague-Dawley rats (approximately 30g, Charles River, Canada) were made diabetic by streptozotocin administration on each of two consecutive days. Onset of diabetes was confirmed by an elevation in urine glucose levels. One half of the animals served as unimmunized controls and received 100 µl PBS/BSA (15 mg/ml) by intravenous (i.v.) injection in the tail vein. The remaining fifty animals were passively immunized by injection of 100 µl of PBS containing

1.5 mg purified IgG anti-PS-ADH-FLA. Ten animals in each of the control and immunized groups were inoculated intraperitoneally (i.p.) with one of five different doses of *B. pseudomallei* 316c: $3x10^3$, $3x10^4$, $3x10^5$, $3x10^6$, $3 \times 10^7$. Animals were monitored carefully for 8 consecutive days for signs of mortality. $LD_{50}$ values were calculated according to Reed and Muench (66).

Table 1. Bacterial strains used in conjugate vaccine studies.

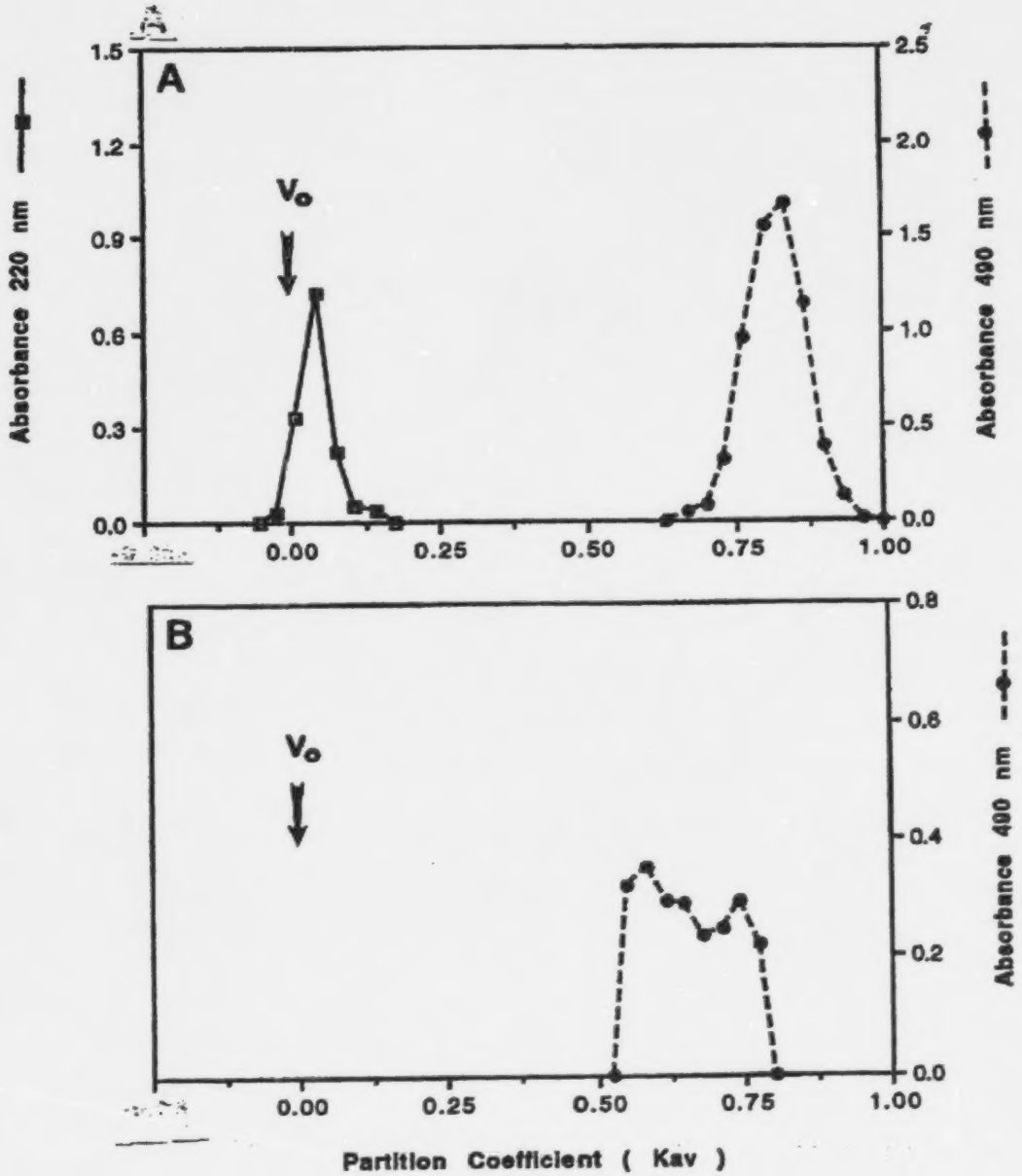
| Strain | Description | Source/Reference |
|---|---|---|
| *P. aeruginosa* | | |
| PAO | Prototroph | |
| *B. pseudomallei* | | |
| 199a | Clinical isolate | D. A. B. Dance |
| 316c | Clinical isolate | D. A. B. Dance |
| 319a | Clinical isolate | D. A. B. Dance |
| 415e | Clinical isolate | D. A. B. Dance |
| 420a | Clinical isolate | D. A. B. Dance |

## C. RESULTS

**Conjugate synthesis.**

In this study the structural and immunological characteristics of three glycoconjuagtes molecules, constructed via the covalent linkage of *B. pseudomallei* 319a PS and flagellin antigens to one another, were examined. The elution profile of the PS-ADH-FLA reaction mixture demonstrated a partition coefficient (Kav) shift in the carbohydrate positive fractions as compared to those of native PS eluted under the same conditions. All fractions testing positive for carbohydrate from the coupling reaction were found to contain protein when assayed by the BCA enhanced protocol method. The absence of native precursor molecules in the column eluates was also demonstrated in the CL-4B fractions (Figure 1). These results are all suggestive of a successful conjugation between the PS and the carrier protein molecules. Of the three conjugates synthesized for these studies, PS-ADH-FLA was the only construct to incorporate ADH as a spacer molecule between the coupled molecular species. The carbohydrate to protein ratio of this conjugate preparation was determined to be 0.88 : 0.12, while the average size of this construct as compared to globular protein standards was $M_r$ = 265,000. The (red/ox)PS-FLA and (ox)PS-FLA glycoconjugates did not require any spacer molecules for their synthesis, and the ratio of carbohydrate to protein in both instances was calculated to be 0.86 : 0.14.

Figure 1. Elution profiles of *B. pseudomallei* 319a flagellar protein, PS and glycoconjugate molecules from Sepharose CL-4B gel permeation chromatography. (A) Flagellar protein (Abs 220 nm: _____) and PS (Abs 490 nm: -------). (B) Elution profile of PS-ADH-FLA glycoconjugate reaction mixture as demonstrated by presence of carbohydrate (Abs 490 nm: -------). $V_o$ represents the void volume of the column.


A
B
$V_o$
$V_o$
Absorbance 220 nm
Absorbance 490 nm
Absorbance 490 nm
1.5
1.2
0.9
0.6
0.3
0.0
2.5
2.0
1.5
1.0
0.5
0.0
0.8
0.6
0.4
0.2
0.0
0.00
0.25
0.50
0.75
1.00
Partition Coefficient ( Kav )

**Immune responses to conjugate molecules.**

The immune serum titres of rabbits immunized with the different conjugate preparations were followed for a period of 42 days post primary immunization. The highest dilution of immune serum that reacted in an ELISA against specific antigen was expressed as the reciprocal value of that dilution (Table 2). In comparison to pre-immune rabbit serum, the PS-ADH-FLA polyclonal antisera clearly demonstrated an increase in IgG titres to both 319a LPS and flagellin proteins after primary immunization. This trend was also shown to continue following the two subsequent booster doses. The (ox)PS-FLA conjugate preparation was the least immunogenic of the three studied. The final titres for LPS and flagellin proteins were 5 fold less than that of (red/ox)PS-FLA with no booster response being observed for LPS between the tertiary dose and day 42. All further experiments requiring anti-conjugate serum were conducted with the PS-ADH-FLA antiserum due to its high titres against both the LPS and flagellin proteins.

**Immunochemical analysis.**

Rabbit polyclonal antiserum raised against the PS-ADH-FLA glycoconjugate preparation was shown to react strongly with both 319a flagellin protein and LPS as demonstrated by Western blot analysis (Figure 2). The immunostaining of the LPS lanes and the adjacent lanes, however, revealed a

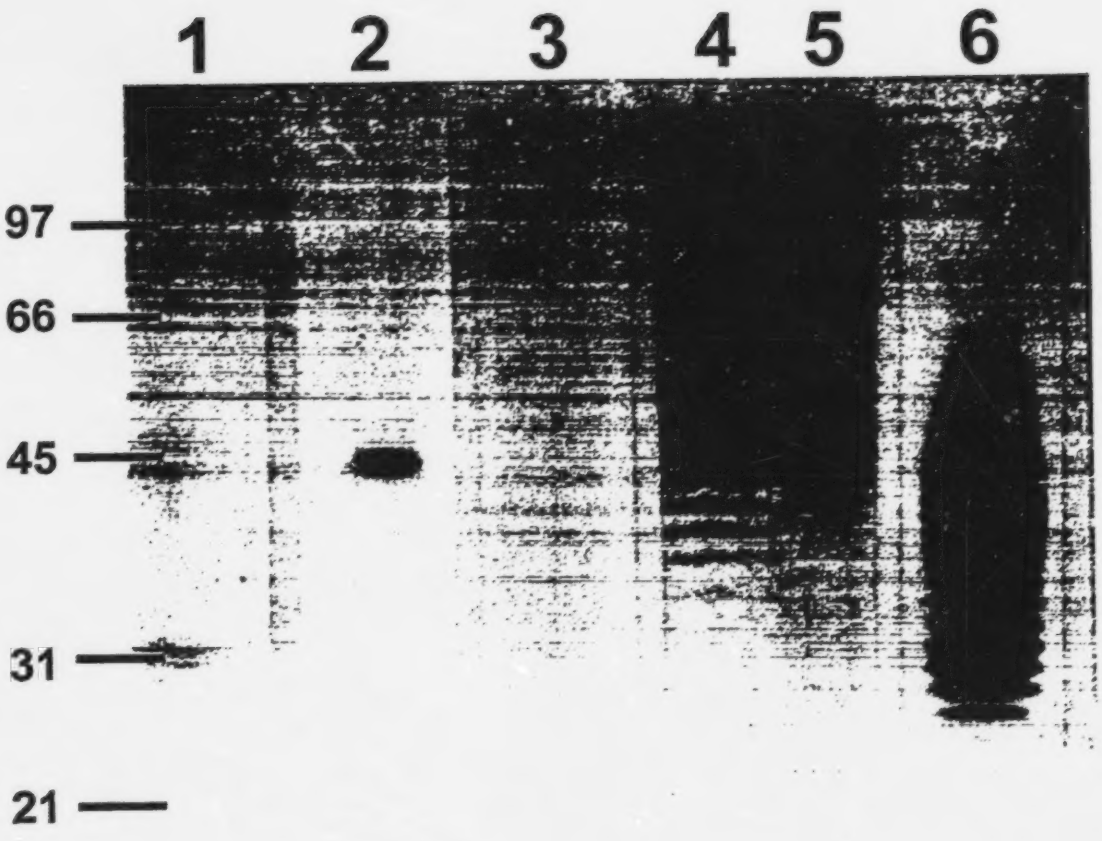
Table 2. Evaluation of the immune responses to three *B. pseudomallei* - specific glycoconjugates in rabbits.

| Immunogen | IgG ELISA titres of immune serum to LPS or FLA on day [a]: | | | | | |
|---|---|---|---|---|---|---|
| | 14 | | 28 | | 42 | |
| | LPS | FLA | LPS | FLA | LPS | FLA |
| PS-ADH-FLA | 625 | 3,125 | 3,125 | 78,125 | 15,625 | 390,625 |
| (red/ox)PS-FLA | ND [b] | ND | 125 | 3,125 | 3,125 | 15,625 |
| (ox)PS-FLA | 125 | 125 | 625 | 625 | 625 | 3,125 |

[a] The ELISA titres of preimmune sera ( n = 2 ) to both LPS and flagellin ( FLA ) obtained at the time of primary immunization ( day 0 ) were < 50. Immune serum samples were collected at 14-day intervals post-primary immunization. Rabbits were given booster injections on days 14 and 28 with Freund's incomplete adjuvant as the emulsifying agent.

[b] ND, not determined for this study.

Figure 2. Western immunoblot analysis of purified *B. pseudomallei* 319a flagellar protein and LPS. Lane 1, standard protein markers were (rabbit muscle phosphorylase b [ 97.4 kDa ], bovine serum albumin [ 66.2 kDa ], hen egg white ovalbumin [ 45.0 kDa ], bovine carbonic anhydrase [ 31.0 kDa ], soybean trypsin inhibitor [ 21.5 kDa ], and hen egg white lysozyme [ 14.4 kDa ]; Bio Rad Laboratories); lane 2, purified flagellar protein (1 μg) reacted with a 1:2000 dilution of PS-ADH-FLA polyclonal antiserum; lane 3, purified flagellar protein (5 μg) reacted with a 1:1000 dilution of *B. pseudomallei* Type II LPS specific MAb; lane 4, LPS antigen migrating from lane E; lane 5, purified LPS (5 μg); lanes D and E reacted with a 1:500 dilution of anti-PS-ADH-FLA polyclonal antiserum; lane 6, purified LPS (3 μg) reacted with a 1:2000 dilution of *B. pseudomallei* Type II LPS specific MAb.


1
2
3
4
5
6
97
66
45
31
21

curious phenomenon. A distinct banding pattern was always found to reside in the wells adjacent to that which had been loaded, while the initially loaded lane displayed a more characteristic smear like pattern (these results were obtained whether the SDS-PAGE gels were loaded with a current running or not). Reaction of the flagellin and LPS samples with the Type II LPS specific MAb demonstrated no reactivity with the purified flagellin proteins and reactivity with only one of the two distinct types of LPS patterns. Lanes adjacent to those loaded with LPS did not react with the MAb as they did with the antibodies directed against the conjugate. Such results suggest that the conjugate antiserum is capable of recognizing both the Type I and II PS antigens of *B. pseudomallei* LPS. The reason as to why the Type I LPS migrates from the wells during electrophoresis is currently being investigated. Immuno-dot blot analysis of 59 *B. pseudomallei* clinical with the PS-ADH-FLA polyclonal rabbit serum demonstrated reactivity with 59/59 of the isolates along with reactivity against *Burkholderia thailandensis* E264 and the PS-ADH-FLA positive control. No reactivity was observed with *E. coli* UB5201, *Burkholderia cepacia* K56-2, *Salmonella typhimurium* 140283 and *P. aeruginosa* PAO (Figure 3 and Table 3). Analysis of the same samples with the Type II LPS specific MAb demonstrated a similar reactivity profile (data not shown).

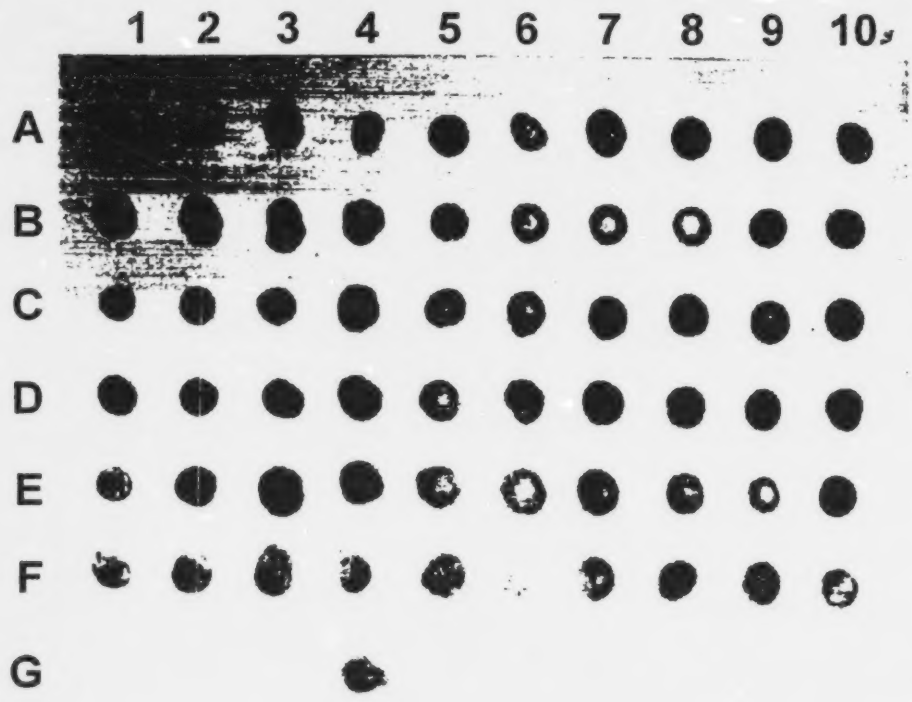



Figure 3. Dot blot analysis of 59 *B. pseudomallei* clinical isolates with PS-ADH-FLA polyclonal antiserum. Well A8 was *B. thailandensis* E264, well G4 was the PS-ADH-FLA conjugate positive control and wells G7-10 were the negative controls *E. coli* UB5201, *B. cepacia* K56-2, *S. typhimurium* 140283 and *P. aeruginosa* PAO respectively. The remaining wells A1-10 through F1-10 were *B. pseudomallei* isolates.

Table 3. Bacterial strains and clinical isolates used in immuno-dot blot and motility inhibition analyses of PS-ADH-FLA polyclonal rabbit antiserum.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| A | *B. p.*[a] 199a | *B. p.* 199b | *B. p.* 230 | *B. p.* 231a | *B. p.* 231/ M1 | *B. p.* 244 | *B. p.* 244a | *B. t.*[b] E264 | *B. p.* 264b | *B. p.* 293a |
| B | *B. p.* 293a /M4 | *B. p.* 303a | *B. p.* 303a | *B. p.* 303d | *B. p.* 304f | *B. p.* 305a | *B. p.* 305d | *B. p.* 305/ M1 | *B. p.* 307a | *B. p.* 307e |
| C | *B. p.* 316c | *B. p.* 319a | *B. p.* 342a | *B. p.* 365a | *B. p.* 365b | *B. p.* 375a | *B. p.* 390a | *B. p.* 309b | *B. p.* 309d | *B. p.* 392 |
| D | *B. p.* 392f | *B. p.* 393/ M4 | *B. p.* 399b | *B. p.* 399c | *B. p.* 402a | *B. p.* 402g | *B. p.* 403a | *B. p.* 405a | *B. p.* 406a | *B. p.* 406e |
| E | *B. p.* 406e | *B. p.* 415a | *B. p.* 415b | *B. p.* 415c | *B. p.* 415d | *B. p.* 415e | *B. p.* 420a | *B. p.* 422a | *B. p.* 422d | *B. p.* 422f |
| F | *B. p.* 424b | *B. p.* 438a | *B. p.* 438c | *B. p.* 443c | *B. p.* 513 | *B. p.* 516 | *B. p.* NCTC 8707 | *B. p.* U7/ mel3 | *B. p.* ? | *B. p.* ? |
| G | X | X | X | PS-ADH-FLA[c] | X | X | *E. c.*[d] UB5201 | *B. p.*[e] K56-2 | *S. t.*[f] 140283 | *P. a.*[g] PAO |

[a] *B. pseudomallei* ( *B. p.* ); [b] *B. thailandensis* ( *B. t.* ); [c] PS-ADH-FLA ( conjugate molecule, 5 μg ); [d] *E. coli* ( *E. c.* ); [e] *B. cepacia* ( *B. c.* ); [f] *S. typhimurium* ( *S. t.* ); [g] *P. aeruginosa* ( *P. a.* ); X designates no antigen present.

**Motility inhibition assay.**

To determine the functional activity of the anti-PS-ADH-FLA antiserum, we examined the ability of this antiserum to inhibit the motility of 59 *B. pseudomallei* isolates (199a, 316c, 319a, 415e, and 420a are displayed in Figure 4 and Table 3). Specificity was examined by testing the ability of this antiserum to inhibit the motility of *P. aeruginosa* PAO (Figure 4). The PS-ADH-FLA antiserum at a 1/100 dilution was shown to inhibit or attenuate the motility of 56/59 isolates tested via this method, and was comparable in efficacy to a 1/100 dilution of anti-319a flagellin antiserum which has previously be shown to inhibit the motility of 64/64 *B. pseudomallei* clinical isolates (9). Neither of the two antisera which inhibited the *B. pseudomallei* isolates were able to inhibit the motility of *P. aeruginosa* PAO or *S. typhimurium* 140283 and *B. cepacia* K56-2. The Type II LPS specific MAb was ineffective in inhibiting the motility of either of the two bacterial species. These results demonstrate that the PS-ADH-FLA antiserum is both functional and specific.

**Passive immunization studies.**

Diabetic rats were inoculated with varying amounts of *B. pseudomallei* 316c i.p. and administered either 1.5 mg of MAPS® II purified anti-319a PS-ADH-FLA IgG in a PBS solution or a BSA/PBS control solution i.v. Of the rats

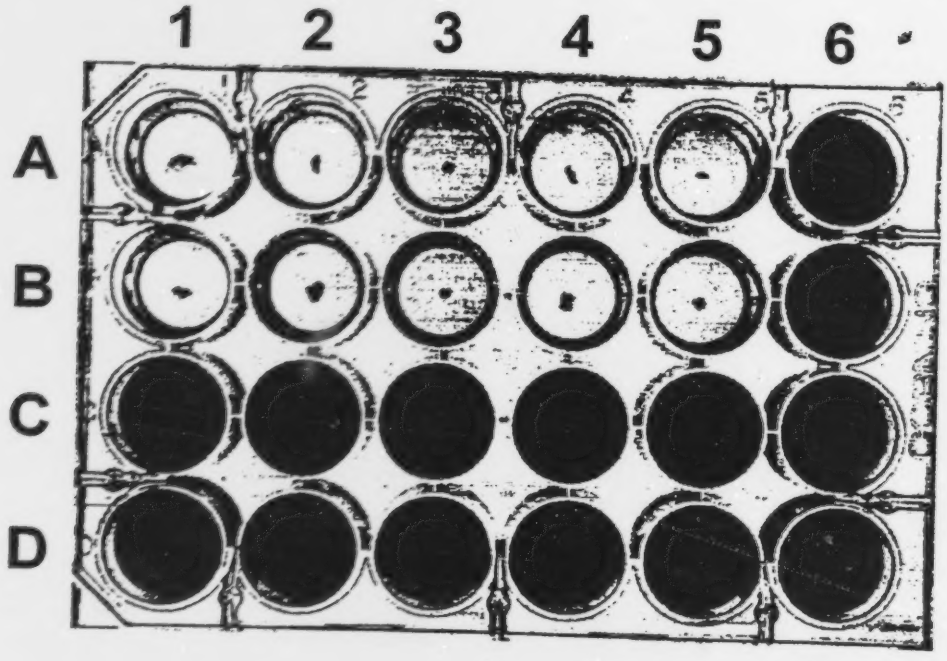



Figure 4. Motility inhibition assessment of selected *B. pseudomallei* strains and *P. aeruginosa* PAO reacted with various *B. pseudomallei* specific antiserum samples. Row A contains a 1:100 dilution of anti-319a flagella specific polyclonal antiserum. Row B contains a 1:100 dilution of anti-PS-ADH-FLA polyclonal antiserum, while row C contains a 1:100 dilution of *B. pseudomallei* Type II LPS MAb. Row D is a control lane with a 1:100 dilution of PBS. Lanes were inoculated as follows: (1) *B. pseudomallei* 199a; (2) *B. pseudomallei* 316c; (3) *B. pseudomallei* 319a; (4) *B. pseudomallei* 415e; (5) *B. pseudomallei* 420a; (6) *P. aeruginosa* PAO.

Table 4. Passive immunoprotection with anti-319a PS-ADH-FLA$_2$ IgG in diabetic rats challenged with *B. pseudomallei* 316c.

| Inoculum size ( CFU/ ml ) | Mortality of diabetic rats of group ( n = 10 ): Control ( BSA i.v. ) [a] | Immunized ( IgG i.v. ) |
|---|---|---|
| $3 \times 10^3$ | 6 | 0 |
| $3 \times 10^4$ | 7 | 0 |
| $3 \times 10^5$ | 7 | 5 |
| $3 \times 10^6$ | 10 | 6 |
| $3 \times 10^7$ | 10 | 9 |

[a] The $LD_{50}$ determined for the control group was $7.54 \times 10^5$ CFU. That of the immunized group was $8.89 \times 10^5$ CFU which was significantly lower than the control ( $P < 0.001$ by chi-square analysis ).

in the PBS control groups, only 20% were still alive after the eighth day; whereas, the immunized groups displayed a 60% survival rate over the same time frame. The most significant results were found in the $3 \times 10^3$ and the $3 \times 10^5$ inoculum groups. The calculated $LD_{50}$ value for the control group was $7.54 \times 10^3$, while that for the immunized group was $8.89 \times 10^5$ (Table 3).

## D. DISCUSSION

While both adults and children often respond favorably to immunization with TD antigens, children are inherently incapable of mounting protective humoral responses against classical TI-2 antigens such as polysaccharides. This defect is the result of ontogenitcally immature immune systems (74, 75). An unfortunate consequence of this TI-2 unresponsiveness is that children are at particular risk of acquiring life threatening illnesses from encapsulated bacterial pathogens such as *Haemophilus influenzae* and *Neisseria meningitidis*. The inability of young children to respond to TI-2 moieties is a complex issue, but is thought to be related to the presence of a defective B cell mIg signaling apparatus and the absence of B lymphocyte stimulation by NK derived cytokines (74, 75). Recently, studies have demonstrated that neonatal B cells are quite capable of secreting protective levels of antibodies against TI-2 antigens if they are presented with the appropriate stimuli. In fact, the addition of cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon-$\gamma$ (IFN-$\gamma$) to TI-2 antigen activated neonatal B cells *in vitro* has been shown to rectify this problem (74).

Since proteinacious vaccines have typically worked well in children, an alternative approach to stimulating neonatal B cells has been to convert TI-2 antigens into TD antigens (20).Thus, the rational behind the construction of glycoconjugate molecules has primarily been to enhance the efficacy of carbohydrate based vaccines in TI-2 antigen unresponsive populations such

as the young. The lessons learned from the construction of these childhood vaccines, however, has also offered an innovative method for the construction of vaccines against bacterial infections for which there are currently none available (20, 40, 41).

In this study, we successfully constructed three PS-flagellin protein glycoconjugates in order to assess the efficacy of these preparations for prophylaxis against *B. pseudomallei* infections. The major focus was to explore whether or not a bivalent preparation offered advantages over the previously characterized monovalent vaccine candidates and to assess the impact of the various coupling procedures on the immunogenicity of the glycoconjugate molecules. The synthesis of these conjugates was based on well defined methods that have been utilized for the construction and examination of a variety of bacterial glycoconjugate molecules.

*B. pseudomallei* strains coordinately express two distinct types of O-antigen on their cell surfaces. The Type I PS antigen is an unbranched homopolymer consisting of 1,3-linked 2-*O*-acetyl-6-deoxy-β-*D*-*manno*-heptopyranose residues. The Type II moiety is an unbranched disaccharide heteropolymer with the structure -3)-β-*D*-glucopyranose-(1-3)-6-deoxy-α-*L*-talopyranose-(1 in which the 6-deoxy-α-*L*-talopyranosyl residues also demonstrate the presence of both *O*-methyl and *O*-acetyl substitutions (48, 64). Interestingly, the presence of the Type II antigen appears to be a critical factor in conferring a serum resistance phenotype on this bacterial pathogen since

mutants lacking this carbohydrate moiety are exquisitely sensitive to the bactericidal effects of normal human serum (18).

The size of the saccharide components displayed by a conjugate molecule appears to greatly influence the immunogenicity of the preparation. In this study, a variety of O-antigen chain lengths were covalently linked to the protein carrier in order to acquire the desired immunological responses associated with PS and oligosaccharide (OS) fragments (21, 22, 41). The incorporation of larger PS fragments acts to increase the size of the conjugate molecules such that the cross-linking of B cell mIg's is maximized. At the same time, the larger PS molecules also tend to retain the conformationally stabilized epitopes which appear to be critical in the design of a successful vaccine (41). The smaller OS fragments appear to elicit immunoglobulin responses against terminal epitopes of the carbohydrate molecules. Recently, an examination of *S. paratyphi* A conjugate vaccines demonstrated that the *O*-acetyl groups present in the native Type A PS antigen are essential for eliciting bactericidal antibody responses (50). Based on these studies, it will also be interesting to determine to what extent the acetylation of *B. pseudomallei* O-antigens effects the immunogenicity of the flagellin-PS conjugates.

By utilizing the flagellin proteins as the carrier molecules, the conjugates coordinately displayed two protective antigens in the same molecule. This is a desirable attribute for a vaccine to possess since it decreases the likelihood that the organism may evade host immunoglobulin responses due to the loss of or a mutation in one of the two protective antigens. Also, the use of a flagellin

protein carrier addresses possible concerns related to the phenomenon of antigen induced immunotolerance (35, 62, 71, 72). It has been proposed that by repeatedly constructing and administering various glycoconjugates with TD carrier molecules such as tetanus toxoid and diphtheria toxoid (11, 15, 27), tolerization of immune systems to these antigens might occur. While this phenomenon is a genuine source for concern, there is conflicting evidence regarding this matter. Further studies will no doubt be required to resolve this matter.

The three glycoconjugates synthesized in this study were shown to exhibit similar carbohydrate/protein ratios even though constructed by differing methods. The saccharide content of these conjugates was 86-88% while the protein content was 12-14%. All three of the reaction mixtures demonstrated the absence of precursor molecules upon completion of the coupling reactions, and the yield of each conjugate preparation was found to be approximately 45-50%. The remaining mass balance of the reaction mixtures was found as insoluble precipitates that were removed by low speed centrifugation prior to the gel filtration step. It thus appears that the synthesis of *B. pseudomallei* PS-flagellin protein molecules is consistent, at least on a structural level, irrespective of the coupling procedures examined to date.

The results of rabbit immune serum titres suggests that method of PS activation, derivatization, and the covalent linkage of the modified PS fragments to the carrier proteins does play an essential role in determining the immunological profiles of the conjugate molecules. Since identical PS

fragment sizes and carrier proteins were used in all three conjugation experiments, these molecules could be eliminated as sources of deviation between the immune titres. Whether one or more of the steps required for the coupling procedures were responsible for these differences is yet to be determined, and further studies will be required to assess this issue. There is no doubt, however, that the PS-ADH-FLA construct was superior in its ability to elicit high titre serum to both LPS and flagellin protein molecules, as compared to (ox)PS-FLA and (red/ox)PS-FLA and that the anamnestic response observed following the booster doses suggests a conversion of the TI-2 PS molecules into TD antigens. Current studies are underway to examine the effects of PS fragment length on the immunogenicity of this glycoconjugate, as well as the ability of these constructs to evoke similar immunoglobulin titres when administered in conjunction with various adjuvants. Also, a more thorough analysis of the evolution of the antibody isotype and sub-class titres needs to be studied following primary and booster immunizations.

The immune serum generated against the PS-ADH-FLA conjugate reacted with both purified flagellin protein and LPS as demonstrated by Western immunoblot analysis. This confirms that at least some of the native epitopes displayed by the precursor molecules are maintained during the construction of the conjugate molecule, a situation that is obviously critical if the immunoglobulin response is to be effective against a *B. pseudomallei* challenge (41, 50). The presence of two unique LPS banding patterns recognized by the PS-ADH-FLA antiserum suggests that both the Type I and II

PS antigens are recognized by the immune serum (48, 64). The more prominent banding profile, which was the result of antigen migration from a loaded well to adjacent wells (even when loaded with an electrophoretic current) is most likely the Type I antigen. This conclusion is substantiated by the inability of the Type II specific anti-LPS monoclonal to react with this particular component. The reasons why only this specific antigen migrates from the well of the SDS-PAGE gels as it does has yet to be fully explained. Immuno-dot blot analysis of 59 *B. pseudomallei* isolates with the conjugate serum demonstrated reactivity with all 59 isolates and suggests that the immune serum should be effective for protecting against numerous *B. pseudomallei* isolates.

The functional properties of the PS-ADH-FLA antiserum were examined in motility inhibition studies with a number of *B. pseudomallei* isolates. At a 1/100 dilution of the immune serum, 56/59 of the strains were completely immobilized while the motility of 3/59 of the strains was partially inhibited. This demonstrates that the flagellin proteins retain critical epitopes responsible for inhibition of flagellar function even when conjugated to the PS molecules. One potential concern of synthesizing the conjugates was the loss of conformational epitopes present only in polymerized flagellin preparations. Although these epitopes may be responsible for eliciting immobilizing antibodies, their absence from the PS-ADH-FLA conjugate did not appear to severely attenuate the immobilizing functionality of the antiserum (at least for the 56/59 strains which were completely immobilized). This concern, however,

may account for the reasons underlying the partial inhibition of *B. pseudomallei* isolates 390a, 390b and 390d. The antiserum was also demonstrated to be species specific in that it was able to inhibit only the motility of *B. pseudomallei* isolates and not *S. typhimurium* 140283, *P. aeruginosa* PAO and *B. cepacia* K56-2.

Passive immunoprotection studies utilizing the purified IgG fraction from the PS-ADH-FLA antiserum have demonstrated that this fraction conferred a level of resistance to a *B. pseudomallei* challenge in the diabetic infant rat model. While the level of protection is less than previously described for the Type II specific MAb and the 319a antiflagellin polyclonal antiserum (9, 11), a number of factors might account for this anomaly. The absence of conformational epitopes in the conjugate preparations may explain a loss in protective capacity in the conjugate antiserum as compared to antiserum raised against polymerized flagellin. The 2.7 fold difference in antibody titres against the flagellin proteins, as demonstrated by the PS-ADH-FLA and 319-specific flagellin antisera, would also be expected to play a significant role in protective capacities of these antisera (9). Also, the Type II LPS specific MAb was an IgM isotype and these immunoglobulins would not be present in the purified IgG fraction used for the protection in our studies. It may well be that IgM isotypes are a significant protective feature associated with humoral immune responses directed against somatic O-antigens expressed by *B. pseudomallei* strains.

In this study, we have demonstrated that the conjugation of *B. pseudomallei* PS to flagellin proteins results in an immunogenic glycoconjugate preparation. The construct has demonstrated the retention of antigen specific and protective epitopes associated the precursor molecules along with the ability to elicit TD type immunoglobulin responses to the normally TI-2 PS antigens. Since *B. pseudomallei* strains have been shown to express both the Type I and II PS antigens (all except 824a which displays only the Type I antigen) and the flagellin proteins appear to be highly conserved on an immunological basis (11, 64), we expect the PS-ADH-FLA conjugate molecule to afford protection against the majority of *B. pseudomallei* strains that we will encounter in our future studies.

## REFERENCES

1. **Aizawa, S.** 1996. Flagellar assembly in *Salmonella typhimurium*. Mol. MicrobioL. **19**(1):1-5.

2. **Akerley, B. J., and J. F. Miller.** 1993. Flagellin gene transcription in *Bordetella bronchiseptica* is regulated by the BvgAS virulence control system. J. Bacteriol. **175**(11):3468-3479.

3. **Allison, J. S., M. Dawson, D. Drake, and T. C. Montie.** 1985. Electrophoretic separation and molecular weight characterization of *Pseudomonas aeruginosa* H-antigen flagellins. Infection and Immunity. **49**(3):770-774.

4. **Atsumi, T., L. McCarter, and Y. Imae.** 1992. Polar and lateral flagellar motors of marine *Vibrio* are driven by different ion-motive forces. Nature. **355**:182-184.

5. **Bahrani, F. K., D. E. Johnson, D. Robbins, and H. L. T. Mobley.** 1991. *Proteus mirabilis* flagella and MR/P fimbriae: isolation, purification, N-terminal analysis, and serum antibody response following experimental urinary tract infection. Infection and Immunity. **59**(10):3574-3580.

6. **Belas, R., and D. Flaherty.** 1994. Sequence and genetic analysis of multiple flagellin-encoding genes from *Proteus mirabilis*. Gene. **148**(1):33-41.

7. **Braley-Mullen, H.** 1980. Antigen requirements for priming of IgG producing B memory cells specific for Type III pneumococcal polysaccharide. Immunology. **40**:521-527.

8. **Brett, P. J., D. DeShazer, and D. E. Woods.** 1997. Characterization of *Burkholderia pseudomallei* and *Burkholderia pseudomallei*-like strains. Epidemiology and Infection. **118**:137-148.

9. **Brett, P. J., D. C. Mah, and D. E. Woods.** 1994. Isolation and characterization of *Pseudomonas pseudomallei* flagellin proteins. Infection & Immunity. **62**(5):1914-1919.

10. **Brunham, R. C., F. A. Plummer, and R. S. Stephens.** 1993. Bacterial antigenic variation, host immune response, and pathogen-host coevolution. Infection and Immunity. **61**(6):2273-2276.

11. **Bryan, L. E., S. Wong, D. E. Woods, D. A. B. Dance, and W. Chaowagul.** 1994. Passive protection of diabetic rats with antisera specific for the polysaccharide portion of the lipopolysaccharide isolated from *Pseudomonas pseudomallei*. Canadian Journal Of Infectious Diseases. **5**:170-178.

12. **Catty, D., and C. Raykundalia.** 1988. Gel immunodiffusion, immunoelectrophoresis and immunostaining methods, p. 164-167. *In* D. Catty (ed.), Antibodies: a practical approach, vol. 1. IRL Press, Washington DC.

13. **Chaowagul, W., N. J. White, D. A. Dance, Y. Wattanagoon, P. Naigowit, T. M. Davis, S. Looareesuwan, and N. Pitakwatchara.** 1989. Melioidosis: a major cause of community-acquired septicemia in northeastern Thailand. Journal of Infectious Diseases. **159**(5):890-899.

14. **Cryz, S. J., Jr., E. Furer, J. C. Sadoff, and R. Germanier.** 1986. *Pseudomonas aeruginosa* immunotype 5 polysaccharide-toxin A conjugate vaccine. Infection and Immunity. **52**:161-165.

15. **Cryz, S. J., Jr., J. C. Sadoff, E. Furer, and R. Germanier.** 1986. *Pseudomonas aeruginosa* polysaccharide-tetanus toxoid conjugate vaccine: safety and immunogenicity in humans. Journal of Infectious Diseases. **154**(4):682-688.

16. **Dar, L., R. Thakur, and V. S. Dar.** 1994. India: is it plague? Lancet. **344**(8933):1359.

17. **Dean, P. G. D., S. W. Johnson, and F. A. Middle.** 1987. Activation Procedures, p. 31-59. *In* P. G. D. Dean, S. W. Johnson, and F. A. Middle (ed.), Affinity chromatography: a practical approach. IRL Press, Oxford.

18. **DeShazer, D., P. Brett, and D. E. Woods.** The Type II O-antigen moiety of Burkholderia pseudomallei lipopolysaccharide is required for serum resistance and virulence. unpublished results.

19. **DeShazer, D., P. J. Brett, R. Carlyon, and D. E. Woods.** 1997. Mutagenesis of *Burkholderia pseudomallei* with Tn5-OT182: Isolation of motility mutants and molecular characterization of the flagellin structural gene. J. Bacteriol.:in press.

20. **Dintzis, R.** 1992. Rational design of conjugate vaccines. Pediatric Research. **32**:376-385.

21. **Dintzis, R. Z., M. H. Middleton, and H. M. Dintiz.** 1985. Inhibition of anti-DNP antibody formation by high doses of DNP-polysaccharide molecules: effect of hapten density and valence. Journal of Immunology. **143**:423-427.

22. **Dintzis, R. Z., M. H. Middleton, and H. M. Dintzis.** 1983. Studies on the immunogenicity and tolerance of T-independant antigens. Journal of Immunology. **131**:2196-2203.

23. **Doig, P., N. Kinsella, P. Guerry, and T. J. Trust.** 1996. Characterization of a post-translational modification of *Campylobacter* flagellin: identification of a sero-specific glycosyl moiety. Molecular Microbiology. **19**:379-387.

24. **Drewry, T. D., G. W. Gray, and S. G. Wilkinson.** 1971. Release of ethanolamine pyrophosphate during mild acid hydrolysis of the lipopolysaccharide of *Pseudomonas aeruginosa*. European Journal of Biochemistry. **21**:400-403.

25. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colormetric method for determination of sugars and related substances. Annals of Chemistry. **28**:350-356.

26. **Esselman, M. T., and P. V. Liu.** 1961. Lecithinase production by gram-negative bacteria. J. Bacteriol. **81**:939-945.

27. **Fattom, A., C. Lue, S. C. Szu, J. Mestecky, G. Shiffman, D. Bryla, W. F. Vann, D. Watson, L. M. Kimzey, J. B. Robbins, and R. Schneerson.** 1990. Serum antibody response in adult volunteers elicited by injection of *Streptococcus pneumoniae* type 12F polysaccharide alone or conjugated to diphtheria toxoid. Infection and Immunity. **58**:2309-2312.

28. **Fattom, A., R. Schneerson, D. C. Watson, W. W. Karakawa, D. Fitzgerald, I. Pastan, X. Li, J. Shiloach, D. A. Bryla, and J. B. Robbins.** 1993. Laboratory and clinical evaluation of conjugate vaccines composed of

*Staphylococcus aureus* Type 5 and Type 8 capsular polysaccharides and bound to *Pseudomonas aeruginosa* recombinant exoprotein A. Infection and Immunity. **61:**1023-1032.

29. **Gold, R., M. L. Lepow, I. Goldschneider, and E. C. Gotschilich.** 1977. Immune response of human infants to polysaccharide vaccines of group A and C *Neisseria meningitidis*. Journal of Infectious Diseases. **136:**S31-35.

30. **Guerry, P., P. Doig, R. A. Alm, D. H. Burr, N. Kinsella, and T. J. Trust.** 1996. Identification and characterization of genes required for post-translational modificaton of *Campylobacter* coli VC167 flagellin. Molecular Microbiology. **19:**369-378.

31. **Guerry, P., S. M. Logan, S. Thornton, and T. J. Trust.** 1990. Genomic organization and expression of *Campylobacter* flagellin genes. Journal of Bacteriology. **172**(4):1853-1860.

32. **Harshey, R. M., and A. Toguchi.** 1996. Spinning tails: homologies among bacterial flagellar systems. Trends in Microbiology.

33. **Heckly, R. J.** 1964. Differentiation of exotoxin and other biologically active substances in *Pseudomonas pseudomallei* filtrates. J. Bacteriol. **88**(6):1730-1736.

34. **Heckly, R. J., and C. Nigg.** 1958. Toxins of *Pseudomonas pseudomallei* II. Characterization. J. Bacteriol. **76:**427-436.

35. **Herzenberg, L. A., T. Tokuhisa, and L. A. Herzenberg.** 1980. Carrier priming leads to hapten-specific suppression. Nature. **285:**665-666.

36. **Heuner, K., L. Bender-Beck, B. C. Brand, P. C. Luck, K. H. Mann, R. Marre, M. Ott, and J. Hacker.** 1995. Cloning and genetic characterization of the flagellum subunit gene (*flaA*) of *Legionella pneumophila* serogroup 1. Infection & Immunity. **63**(7):2499-2507.

37. **Hodgkin, P. D., and A. Basten.** 1995. B cell activation, tolerance and antigen-presenting function. Current Opinion in Immunology. **7**:121-129.

38. **Howe, C., A. Sampath, and M. Spotnitz.** 1971. The pseudomallei group: a review. Journal of Infectious Diseases. **124**(6):598-606.

39. **Insel, R. A., and P. Anderson.** 1986. Oligosaccharide-protein conjugate vaccines induce and prime for oligoclonal IgG antibody responses to *Haemophilus influenzae* b capsular polysaccharide in human infants. Journal of Experimental Medicine. **163**:262-269.

40. **Jennings, H.** 1983. Capsular polysaccharides as human vaccines. Advances in Carbohydrate Chemistry. **41**:155-208.

41. **Jennings, H.** 1992. Further approaches for optimizing polysaccharide-protein conjugate vaccines for prevention of invasive bacterial disease. Journal of Infectious Diseases. **165**:S156-159.

42. **Jennings, H., and C. Lugowski.** 1981. Immunochemistry of groups A, B and C meningococcal polysaccharide-tetanus toxoid conjugates. Journal of Immunology. **127**:1011-1018.

43. **Johnson, K. G., and M. B. Perry.** 1975. Improved techniques for the the preparation of bacterial lipopolysaccharides. Canadian Journal of Microbiology. **22**:29-34.

44. **Joys, T. M.** 1988. The flagellar filament protein. Canadian Journal of Microbiology. **34**(4):452-458.

45. **Kanto, S., H. Okino, S. Aizawa, and S. Yamaguchi.** 1991. Amino acids responsible for flagellar shape are distributed in terminal regions of flagellin. Journal of Molecular Biology. **219**(3):471-480.

46. **Kelly-Wintenberg, K., T. Anderson, and T. C. Montie.** 1990. Phosphorylated tyrosine in the flagellum filament protein of *Pseudomonas aeruginosa*. Journal of Bacteriology. **172**(9):5135-5139.

47. **Kelly-Wintenberg, K., S. L. South, and T. C. Montie.** 1993. Tyrosine phosphate in a- and b- Type flagellins of *Pseudomonas aeruginosa*. Journal of Bacteriology. **175**(8):2458-2461.

48. **Knirel, Y. A., N. A. Paramonov, A. S. Shashkov, N. K. Kochetkov, R. G. Yarullin, S. M. Farber, and V. I. Efremenko.** 1992. Structure of the polysaccharide chains of *Pseudomonas pseudomallei* lipopolysaccharides. Carbohydrate Research. **233**:185-193.

49. **Konadu, E., J. B. Robbins, J. Shiloach, D. B. Bryla, and S. C. Szu.** 1994. Preparation, charaterization and immunological properties in mice of *Escherichia coli* O157 O-specific polysaccharide-protein conjugate vaccines. Infection and Immunity. **62**:5048-5054.

50. **Konadu, E., J. Shiloach, D. A. Bryla, J. B. Robbins, and S. C. Szu.** 1996. Synthesis, charaterization, and immunological properties in mice of conjugates composed of detoxified lipopolysaccharide of *Salmonella paratyphi* A bound to

tetanus toxoid, with emphasis on the role of O acetyls. Infection and Immunity. **64**(7):2709-2715.

51. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. **227**:680-685.

52. **Leelarasamee, A., and S. Bovornkitti.** 1989. Melioidosis: review and update. Reviews of Infectious Diseases. **11**(3):413-425.

53. **Lett, E., S. Gangloff, M. Zimmerman, D. Wachsmann, and J.-P. Klein.** 1994. Immunogenicity of polysaccharides conjugated to peptides containing T- and B-cell epitopes. Infection and Immunity. **62**:785-792.

54. **Logan, S. M., L. A. Harris, and T. J. Trust.** 1987. Isolation and charaterization of *Campylobacter* flagellins. Journal of Bacteriology. **169**:5072-5077.

55. **Macnab, R. M., and D. J. DeRosier.** 1988. Bacterial flagellar structure and function. Canadian Journal of Microbiology. **34**(4):442-451.

56. **Milton, D. L., R. O'Toole, P. Horstedt, and H. Wolf-Waltz.** 1996. Flagellin A is essential for the virulence of *Vibrio anguillarum*. J. Bacteriol. **178**(5):1310-1319.

57. **Mond, J. J., Q. Vos, A. Lees, and C. M. Snapper.** 1995. T cell independent antigens. Current Opinions in Immunology. **7**:349-354.

58. **Montie, T. C., R. C. Craven, and I. A. Holder.** 1982. Flagellar preparations from *Pseudomonas aeruginosa*: isolation and characterization. Infection and Immunity. **35**:281-288.

59. **Murphy, T. F.** 1994. Antigenic variation of surface proteins as a survival strategy for bacterial pathogens. Trends in Microbiology. **2**(11):427-429.

60. **Nachamkin, I., and X. H. Yang.** 1989. Human antibody response to *Campylobacter jejuni* flagelin protein and a synthetic N-terminal flagellin peptide. Journal of Clinical Microbiology. **27**:2195-2198.

61. **Oiler, L., F. Kocka, R. W. Smith, and H. Koffler.** 1971. The presence of flagellins A and B in the flagellum of *Bacillus pumilus* 101. Bacteriology Proceedings. **1971**:27.

62. **Peeters, C. C. A. M., A.-M. Tenbergen-Meekes, J. T. Poolman, M. Beurret, B. J. M. Zegers, and G. T. Rijkers.** 1991. Effect of carrier priming on immunogenicity of saccharide-protein conjugate vaccines. Infection and Immunity. **59**:3504-3510.

63. **Penn, C. W., and C. J. Luke.** 1992. Bacterial flagellar diversity and significance in pathogenesis. FEMS Microbiology Letters. **100**:331-336.

64. **Perry, M. B., L. L. MacLean, T. Schollaardt, L. E. Bryan, and M. Ho.** 1995. Structural characterization of the lipopolysaccharide O antigens of *Burkholderia pseudomallei*. Infection & Immunity. **63**(9):3348-3352.

65. **Rasiah, C., E. Schiltz, J. Reichert, and A. Voit.** 1992. Purification and characterization of a tryptic peptide of *Borrelia burgdorferi* flagellin, which reduces cross-reactivity in immunoblots and ELISA. Journal of General Microbiology. **141**:2053-2061.

66. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27**(3):493-497.

67. **Robbins, J. B.** 1978. Vaccines for the prevention of encapsulated bacterial diseases: current status, problems and prospects for the future. Immunochemistry. **15**:839-853.

68. **Sambrook, J., E. F. Fritsch, and T. Maniatis (ed.).** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

69. **Schoenhals, G., and C. Whitfield.** 1993. Comparative analysis of flagellin sequences from *Escherichia coli* strains possessing serologically distinct flagellar filaments with a shared complex surface pattern. Journal of Bacteriology. **175**(17):5395-5402.

70. **Sexton, M. M., A. L. Jones, W. Chaowagul, and D. E. Woods.** 1994. Purification and characterization of a protease from *Pseudomonas pseudomallei*. Canadian Journal of Microbiology. **40**(11):903-910.

71. **Shutze, M. P., E. Deriaud, G. Przewlocki, and C. Leclerc.** 1989. Carrier-induced epitopic suppression is initiated through clonal dominance. Journal of Immunology. **142**:2635-2640.

72. **Shutze, M. P., C. Leclerc, M. Jolivet, F. Audibert, and L. Chedid.** 1985. Carrier-induced epitope suppression, a major issue for synthetic vaccines. Journal of Immunology. **135**:2319-2322.

73. **Smith, C. J., J. C. Allen, M. N. Embi, O. Othman, N. Razak, and G. Ismail.** 1987. Human melioidosis: and emerging medical problem. MIRCEN J. **3**:343-366.

74. **Snapper, C. M., and J. J. Mond.** 1996. A model for induction of T cell-independent humoral immunity in response to polysaccharide antigens. Journal of Immunology. **157:**2229-2233.

75. **Snapper, C. M., F. R. Rosa, M. A. Moorman, and J. J. Mond.** 1997. Restoration of T cell-independent type 2 induction of Ig secretion by neonatal B cells *in vitro.* Journal of Immunolgy. **158:**2731-2735.

76. **Steinmetz, I., M. Rohde, and B. Brenneke.** 1995. Purification and characterization of an exopolysaccharide of *Burkholderia* (*Pseudomonas*) *pseudomallei.* Infection & Immunity. **63**(10):3959-3965.

77. **Tamura, Y., M. Kijima-Tanaka, A. Aoki, Y. Ogikubo, and T. Takahashi.** 1995. Reversible expression of motility and flagella in *Clostridium chauvoei* and their relationship to virulence. Microbiology. **141:**605-610.

78. **Totten, P. A., and S. Lory.** 1990. Characterization of the type a flagellin gene from *Pseudomonas aeruginosa* PAK. Journal of Bacteriology. **172**(12):7188-7199.

79. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences of the United States of America. **74:**4350-4354.

80. **Watson, D. C., J. B. Robbins, and S. C. Szu.** 1992. Protection of mice against *Salmonella typhimurium* with an O-specific polysaccharide-protein conjugate vaccine. Infection and Immunity. **60:**4679-4686.

81. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and some applications, p. 83-91. *In* R. L. Whistler (ed.), Methods in Carbohydrate Chemistry, vol. 5. Academic Press, New York.

82. **Wilson, D. R., and T. J. Beveridge.** 1993. Bacterial flagellar filaments and their component flagellins. Canadian Journal of Microbiology. **39**:451-472.

83. **Winstanley, C., and A. W. Morgan.** 1997. The bacterial flagellin gene as a biomarker for detection, population genetics and epidemiological analysis. Microbiology. **143**:3071-3084.

84. **Winter, A. J., G. E. Rowe, J. R. Duncan, M. J. Eis, and B. Widom.** 1988. Effectiveness of natural and synthetic complexes of porin and O-polysaccharide as vaccines against *Brucella abortus* in mice. Infection and Immunity. **56**:2808-2817.

85. **Woods, D. E., A. L. Jones, and P. J. Hill.** 1993. Interaction of insulin with *Pseudomonas pseudomallei*. Infection & Immunity. **61**(10):4045-4050.

86. **Yabuuchi, E., Y. Kosako, H. Oyaizu, I. Yano, H. Hotta, Y. Hashimoto, T. Ezaki, and M. Arakawa.** 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiology & Immunology. **36**(12):1251-1275.

87. **Yang, H., C. D. Kooi, and P. A. Sokol.** 1993. Ability of *Pseudomonas pseudomallei* malleobactin to acquire transferrin-bound, lactoferrin-bound, and cell-derived iron. Infection & Immunity. **61**(2):656-662.

88. **Yang, H. M., W. Chaowagul, and P. A. Sokol.** 1991. Siderophore production by *Pseudomonas pseudomallei*. Infection & Immunity. **59**(3):776-780.

89. **Yao, R., D. H. Burr, P. Doig, T. J. Trust, H. Niu, and P. Guerry.** 1994. Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni* : the role of motility in adherence and invasion of eukaryotic cells. Molecular Microbiology. **14**(5):883-893.